DEVELOPMENT OF STRIP BASED TESTS FOR DETECTION OF SELECTED ADULTERANTS AND INDIGENOUS ENZYMES IN MILK AND MILK PRODUCTS

THESIS SUBMITTED TO THE
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
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IN PARTIAL FULFILLMENT OF THE REQUIREMENT
FOR THE AWARD OF THE DEGREE OF

MASTER OF TECHNOLOGY
IN
DAIRY CHEMISTRY
BY

PRIYAE BRATH GAUTAM
B.Tech. (Dairy Technology)

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Karnal – 132 001 (Haryana), India

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CERTIFICATE

This is to certify that the thesis entitled "**DEVELOPMENT OF STRIP BASED TESTS FOR DETECTION OF SELECTED ADULTERANTS AND INDIGENOUS ENZYMES IN MILK AND MILK PRODUCTS**" submitted by **Mr. PRIYAE BRATH GAUTAM** towards the partial fulfilment of the requirements 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 him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

Dated: 26/7/16

Dr. Rajan Sharma
MAJOR ADVISOR & CHAIRMAN
(GUIDE)

Dedicated to the Supreme Lord of Lords

ABSTRACT

The present study was conducted to develop and validate the rapid methods for the detection of selected adulterants and enzymes in milk. During this study, new strip tests have been developed for the detection of neutralizer, added urea, hydrogen peroxide and sucrose in milk. In addition to this, a lateral flow assay was developed for the detection of soymilk in milk. The strips were also validated in milk products like *khoa* and *dahi*. The test strips were analysed to establish response time, limit of detection, repeatability, reproducibility, probability of false positive/negative, shelf life. In standard conditions, test strips were validated in our own laboratory and it was found that test strips have good repeatability (within a prepared batch of strips) and reproducibility (among different batches of strips), and chances of false positive results are nil in control milk samples. The sensitivity of the strips was established and LOD in milk was 0.03% for NaOH (0.05% Na_2CO_3, 0.1% $NaHCO_3$); 0.08% for urea (total urea); 0.1% for sucrose and 0.001% for H_2O_2. The response time at which a detectable colour change occurred was found immediately for neutralizers and hydrogen peroxide; 3 min for urea and sucrose. The developed strips were easy to use and require just one step wherein either they are dipped in milk sample or a drop of milk sample is applied to the prepared strip followed by visualization of change in colour. In all cases, sensitivity of the prepared strips was found better than the existing test based on wet chemistry. The shelf-life of strips is 8 months at room temperature for neutralizer, 4 months at refrigeration temperature for urea, 4 months for hydrogen peroxide and 1 month for sucrose.

Competitive method was used in LFA for the detection of soymilk. Gold nanoparticles (AuNPs) were prepared by citrate reduction method having average size of ±28.59 nm and zeta potential of (±) -38.3 mV. AuNPs were coupled to anti-soya antibody. The antibody-AuNPs conjugate was applied on the conjugate pad. The test line of lateral flow strip consisted of laboratory made soymilk while the control line of lateral flow strip consisted of secondary antibody (raised in goat against rabbit). The assay was validated with samples of soymilk in borate buffer, skim milk and whole milk and involved application of the samples to the adsorbent pad of the lateral flow strip followed by visualization of appearance of one or two red lines within 5 minute on the nitrocellulose membrane. The detection limit of the soymilk was 1.25%, 1.5% and 1.75% in borate buffer, skim milk and whole milk respectively.

In addition to it, strips for detergent, alkaline phosphatase and lactoperoxidase were also tried to develop but with little or no success. As a result these strips were abandoned from the research work. The strips for glucose, sucrose and maltodextrin (@0.5, 1%) were also validated in *khoa* and *dahi*.

सारांश

दूध में चयनित मिलावट और एंजाइमों का पता लगाने के लिए तीव्र तरीकों का विकास और उन्हे मान्य करने पर कार्य किया गया है । इस अध्ययन के दौरान, नई प्रकार की कागज़ की पट्टियाँ दूध में न्यूट्रालाइज़र्स, यूरिया (कुल यूरिया), हाइड्रोजन पेरोक्साइड और सुक्रोज (साधारण चीनी) का पता लगाने के लिए विकसित की गई है। इन की कागज़ की पट्टियाँ को दूध उत्पाद जैसे खोआ और दही में भी जांचा किया गया । इसके अलावा, लेटरल फ्लो पट्टी दूध में सोया दूध का पता लगाने के लिए विकसित किया गया है। । कागज़ की पट्टियों का प्रतिक्रिया समय, मिलावट पता लगाने की सीमा, रेपीटबिलिटी, रेप्रोदुसिबिलिटी, झूठे सकारात्मक/नकारात्मक संभावना और अचल जीवन का विश्लेषण किया गया । मानक स्थितियों में, कागज़ की पट्टियाँ हमारी अपनी प्रयोगशाला में मान्य किया गया और यह पाया गया कि कागज़ की पट्टियाँ की रेपीटबिलिटी (स्ट्रिप्स के एक तैयार बैच के भीतर) और रेप्रोदुसिबिलिटी (स्ट्रिप्स के विभिन्न बैचों के बीच) अच्छा है और झूठी सकारात्मक परिणाम की संभावना नियंत्रण दूध के नमूने में नहीं के बराबर हैं । कागज़ की पट्टियों की संवेदनशीलता स्थापित किया गया है और दूध में मिलावट पता लगाने की सीमा NaOH के लिए 0.03% (0.05% Na_2CO_3, 0.1% $NaHCO_3$) थी; यूरिया (कुल यूरिया) के लिए 0.08%; सुक्रोज के लिए 0.1%; H_2O_2 के लिए 0.001% थी । प्रतिक्रिया समय जिस पर एक पता लगाने योग्य रंग परिवर्तन हुआ है, न्यूट्रालाइज़र्स और हाइड्रोजन पेरोक्साइड के लिए तुरंत; यूरिया और सुक्रोज के लिए 3 मिनट पाया गया था । विकसित कागज़ की पट्टियाँ उपयोग करने में आसान हैं जिसमें परीक्षण करने के लिए या तो दूध के नमूने की एक बूंद को पट्टी पर डाला जाता है या पट्टी को दूध में डूबा दिया जाता है । सभी मामलों में, तैयार कागज़ की पट्टियाँ की संवेदनशीलता दूध परीक्षण के मौजूदा तरीके जो गीले रसायन विज्ञान के आधार पर काम कर रहे हैं, उनसे बेहतर थी । कागज़ की पट्टियों के शेल्फ जीवन, न्यूट्रालाइज़र के लिए कमरे के तापमान पर 8 महीने है, यूरिया के लिए प्रशीतन के तापमान पर 4 महीने, हाइड्रोजन पेरोक्साइड के लिए 5 महीने और सुक्रोज के लिए 1 महीने तक थी । ग्लूकोज, माल्टोडेक्सट्रिन और सुक्रोज का पता लगाने के लिए पहले से ही विकसित कागज़ की पट्टियों को अध्ययन के दौरान खोआ और दही में जांचा गया। उपभोक्ताओं के स्तर पर कागज़ की पट्टियों के लिए आसान काम करने के लिए, एक प्रोटोकॉल भी विकसित किया गया जिसमें कागज़ की पट्टियों को गोंद से चिपकाने पर पट्टी पर चिपका दिया गया। इसके अलावा, डिटर्जेंट, क्षारीय फॉस्फेट और lactoperoxidase के लिए भी स्ट्रिप्स विकसित करने की कोशिश की गई लेकिन बहुत कम या कोई सफलता ना मिलने पर नतीजतन इन कागज़ की पट्टियों का शोध कार्य रोक दिया गया।

दूध में सोया दूध की मिलावट पता लगाने के लिए लेटरल फ्लो पट्टी में, प्रतिस्पर्धी प्रारूप का प्रयोग किया गया था । श्लैष स्वर्ण नैनोकण (एयूएनपी) सिट्रेट अपचयन पद्धति द्वारा बनाये गए, उन श्लैष स्वर्ण नैनोकणों का औसत आकार 28.59 नैनोमीटर और जीटा विभव -38.3 मिलीवोल्ट था । स्वर्ण नैनोकणों का सोया एंटीबॉडी के साथ संयुग्मन किया गया । स्वर्ण नैनोकणों-सोया एंटीबॉडी संयुग्मन को संयुग्म पैड पर लागू किया गया । पार्श्व प्रवाह पट्टी की परीक्षण लाइन प्रयोगशाला में निर्मित सोया दूध की थी, जबकि नियंत्रण रेखा माध्यमिक एंटीबॉडी (खरगोश के खिलाफ बकरी में उठाई) की थी । सोया दूध के नमूनों को बोरेट बफर, मलाई निकाला हुआ दूध एवं पूरे दूध में मान्य किया गया और नमूना पैड पर लगाया गया, जिसके 5 मिनट पश्चात नाइट्रोसेलुलोज झिल्ली पर एक या दो लाल लाइनों की उपस्थिति दिखाई देने तक प्रतिक्षा प्रतिक्षा की गई । सोया दूध की सीमा बोरेट बफर, मलाई निकाला हुआ दूध एवं पूरे दूध में 1.25%, 1.5% और 1.75% क्रमशः पाई गई ।

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CONTENTS

Chapter	Title	Page No.
1.0	**INTRODUCTION**	**1-3**
2.0	**REVIEW OF LITERATURE**	**4-33**
	2.1 Dry reagent chemistry	4
	2.1.1 Introduction	4
	2.1.2 Past and current activity	4
	2.1.3 Lateral flow assay	9
	2.1.3.1 Principles of Lateral flow assay	9
	2.1.4 Structural components of lateral flow assay strip	10
	2.1.4.1 Membrane material	10
	2.1.4.2 Material of sample pad	11
	2.1.4.3 Material of absorbent pad	11
	2.1.4.4 Conjugate pad	12
	2.1.4.5 Labels for detection	12
	2.1.4.6 Position of the test line	13
	2.1.5 Formats of lateral flow assay	13
	2.1.5.1 Competitive format	13
	2.1.5.2 Sandwich format	14
	2.1.6 Construction of lateral flow strip and analyte detection	14
	2.1.7 Advantages and Disadvantages of LFA	15
	2.1.8 Quantification using dry reagent tests	15
	2.2 Methods for detection of selected adulterants in milk	17
	2.2.1 Detection of hydrogen peroxide	17
	2.2.2 Detection of neutralizers in milk	18
	2.2.2.1 Colorimetric methods	18
	2.2.2.2 Measurement of alkalinity of ash	19
	2.2.2.2.1 Direct titration method for alkalinity of ash	19
	2.2.2.2.2 Back titration method for alkalinity of ash	20

2.2.2.3 Determination of sodium, potassium and calcium 21

2.2.3 Methods for detection and determination of urea in milk 21

2.2.3.1 Direct methods 22

2.2.3.1.1 DMAB method 22

2.2.3.1.2 Diacetyl monoxime (DAM) method 23

2.2.3.2 Indirect methods 23

2.2.3.3 Physical methods 24

2.2.3.3.1 Infrared measurements 24

2.2.3.3.2 Enzymatic methods coupled with pH of conductivity measurements 24

2.2.4 Detection and determination of detergents in milk 25

2.2.4.1 Detection of detergents containing Alkyl Benzene Sulphonic Acid (ABS) 26

2.2.4.2 Detection of detergents in milk using paper chromatography 28

2.2.4.3 Detection of detergents in milk using Bromo cresol purple indicator 28

2.2.5 Detection of sucrose in milk 28

2.2.5.1 Modified Seliwanoff's method 28

2.2.5.2 By high performance liquid chromatography (HPLC) 28

2.2.6 Detection of alkaline phosphatase in milk 28

2.2.7 Detection of lactoperoxidase in milk 29

2.2.7.1 Quantitative enzyme assay 30

2.2.7.2 Spectrophotometric method 30

2.2.8 Detection of soya protein in milk 30

2.2.8.1 Polarimetric method 31

2.2.8.2 Isoelectric precipitation method 31

2.2.8.3 SDS-PAGE method 31

2.2.8.4 Immunodiffusion method 32

2.2.8.5 HPLC based method 32

2.3 Conclusion 32

3.0 MATERIAL AND METHOD 34-54

3.1 Milk samples and other materials 34

3.2 Qualitative detection of added neutralizers in milk using dry reagent strip 34

3.2.1 Chemicals and materials 34

3.2.2 Method of preparation of neutralizer detection strip 34

3.2.2.1 Adsorption of chromogen on a filter paper 34

3.2.3 Proof of principle of working of prepared dry reagent test strip in neutralizer added milk samples 34

3.2.4 Determination of response time 35

3.2.5 Determination of limit of detection of various neutralizers in milk using test strip 35

3.3 Qualitative detection of added urea in milk using dry reagent strip 35

3.3.1 Chemicals and materials 35

3.3.2 Reagents 36

3.3.3 Method of preparation of urea detection strip 36

3.3.3.1 Adsorption of chromogen 1 and 2 on the filter paper 36

3.3.3.2 Adsorption of chromogen 3 on the filter paper pre-adsorbed with chromogen 1 and 2 36

3.3.4 Proof of principle of working of prepared dry reagent strip in urea added milk 36

3.3.5 Determination of response time 37

3.3.6 Determination of limit of detection of urea in milk using prepared test strip 37

3.4 Qualitative detection of added sucrose in milk using dry reagent strip 37

3.4.1 Chemicals and materials 37

- 3.4.2 Reagents 38
- 3.4.3 Method of preparation of sucrose detection strip 38
  - 3.4.3.1 Preparation of enzyme solution 38
  - 3.4.3.2 Preparation of chromogen solution 38
  - 3.4.3.3 Preparation of soaking solution 38
  - 3.4.3.4 Adsorption of reagents on filter paper 38
- 3.4.4 Proof of principle of working of prepared dry reagent strip in sucrose added milk 39
- 3.4.5 Determination of response time 39
- 3.4.6 Determination of limit of detection of sucrose in milk using prepared test strip 39

3.5 Qualitative detection of added hydrogen peroxide in milk using dry reagent strip 39

- 3.5.1 Chemicals and materials 39
- 3.5.2 Method of preparation of hydrogen peroxide detection strip 40
  - 3.5.2.1 Preparation of enzyme solution 40
  - 3.5.2.2 Preparation of chromogen solution 40
  - 3.5.2.3 Preparation of soaking solution 40
  - 3.5.2.4 Adsorption of reagents on filter paper 40
- 3.5.3 Proof of principle of working of prepared dry reagent strip in hydrogen peroxide added milk 40
- 3.5.4 Determination of response time 41
- 3.5.5 Determination of limit of detection of hydrogen peroxide in milk using test strip 41

3.6 Qualitative detection of detergent in milk using dry reagent strip 41

3.6.1 Chemicals and materials 41
3.6.2 Method of preparation of detergent detection strip 41
3.6.2.1 Preparation of chromogen solution 41
3.6.2.2 Preparation of soaking solution 42
3.6.2.3 Adsorption of reagents on filter paper 42
3.6.2.4 Procedure for detection of detergent in milk using detergent detection strip 42
3.7 Qualitative detection of added soymilk by lateral flow assay 42
3.7.1 Chemicals, material and equipments 42
Chemicals
3.7.2 Preparation of Gold nanoparticles (AuNPs) 43
3.7.3 Characterization of Gold nanoparticles 45
3.7.3.1 Measurement of size and zeta potential of AuNPs 45
3.7.3.2 Measurement of size and zeta potential of AuNPs 45
3.7.4 Conjugation of AuNPs with antibody 45
3.7.5 Construction of Lateral Flow Assembly 46
3.7.6 Detection of soymilk in milk using Lateral Flow Assay 48
3.7.6.1 Preparation of soymilk 48
3.8 Detection of alkaline phosphatase in milk by dry reagent chemistry 49
3.8.1 Using BCIP Red/ NBT Solution A 49
3.8.1.1 Chemicals 49
3.8.1.2 Apparatus 49
3.8.1.3 Detection of alkaline phosphatase in liquid phase 49
3.8.1.4 Method of preparation of alkaline phosphatase detection strip 50

- 3.8.1.4.1 Adsorption of reagents on the filter paper 50
- 3.8.1.4.2 Procedure for detection of alkaline phosphatase in milk 50
- 3.8.2 Using phenolphthalein diphosphate tetrasodium salt 50
  - 3.8.2.1 Chemicals 50
  - 3.8.2.2 Apparatus 50
  - 3.8.2.3 Detection of alkaline phosphatase in liquid phase 50
  - 3.8.2.4 Method of preparation of alkaline phosphatase detection strip 51
    - 3.8.2.4.1 Adsorption of reagents on the filter paper 51
    - 3.8.2.4.2 Procedure for detection of alkaline phosphatase in milk 51
- 3.9 Detection of lactoperoxidase in milk by dry reagent chemistry 51
  - 3.9.1 Using TMB liquid substrate for membranes 51
    - 3.9.1.1 Chemicals 51
    - 3.9.1.2 Reagents 51
    - 3.9.1.3 Apparatus 52
    - 3.9.1.4 Detection of lactoperoxidase in liquid phase 52
    - 3.9.1.5 Method of preparation of lactoperoxidase detection strip 52

3.9.1.5.1 Adsorption of reagents on the filter paper 52
3.9.1.5.2 Procedure for detection of lactoperoxidase in milk 52
3.9.2 Using 4-AAP and Phenol 52
3.9.2.1 Chemicals 52
3.9.2.2 Reagents 53
3.9.2.3 Apparatus 53
3.9.2.4 Detection of lactoperoxidase in liquid milk 53
3.9.2.5 Method of preparation of lactoperoxidase detection strip 53
3.9.2.5.1 Adsorption of reagents on the filter paper 53
3.9.2.5.2 Procedure for detection of lactoperoxidase in milk 54
3.10 Validation Of The Test Strips In Milk Products 54
3.10.1 Validation of glucose, sucrose, Maltodextrin detecting paper strips in dahi 54
3.10.1.1 Preparation of dahi spiked with glucose, sucrose and Maltodextrin 54
3.10.2 Validation of glucose, sucrose, Maltodextrin detecting paper strips in khoa. 54
3.10.2.1 Preparation of khoa spiked with glucose, sucrose and Maltodextrin 54

4.0 RESULTS AND DISCUSSION 55-85
4.1 Neutralizer detection strip 55
  4.1.1 Working of neutralizer detection strip 55
  4.1.2 Determination of response time of neutralizer detection strip 56
  4.1.3 Determination of LOD of neutralizer detection strip 56
  4.1.4 Storage study of neutralizer detection strip 58
4.2 Urea detection strip 59
  4.2.1 Working of urea detection strip 59
  4.2.2 Determination of response time of urea detection strip 60
  4.2.3 Determination of LOD of urea detection strip 60
  4.2.4 Storage study of urea detection strip 61
4.3 Sucrose detection strip 61
  4.3.1 Working of sucrose detection strip 61
  4.3.2 Determination of response time of sucrose detection strip 63
  4.3.3 Determination of LOD of sucrose detection strip 64
  4.3.4 Storage study of sucrose detection strip 64
4.4 Hydrogen peroxide detection strip 64
  4.4.1 Working of the hydrogen peroxide strip 64
  4.4.2 Determination of response time of hydrogen peroxide strip 65
  4.4.3 Determination of LOD of hydrogen peroxide strip 65
  4.4.4 Storage study of hydrogen peroxide strip 66
4.5 Detergent detection strip 66
4.6 Detection of soymilk by lateral flow assay 67
  4.6.1 Preparation of gold nanoparticles (AuNPs) and their characterization 68
  4.6.2 Optical properties of AuNPs 68
  4.6.3 Measurement of size and zeta potential of prepared AuNPs using dynamic light scattering (DLS) 68
  4.6.4 Gold nanoparticles-antibody conjugate 71
  4.6.5 Principle of lateral flow strip and test 71

determination
4.6.6 Detection of soymilk using AuNPs conjugated with antibody by lateral flow assay 72
4.7 Alkaline phosphatase detection strip 75
4.7.1 Working of alkaline phosphatase detection strip 75
4.7.1.1 Using BCIP/NBT Solution A 75
4.7.1.2 Using phenolphthalein diphosphate tetrasodium salt 76
4.8 Lactoperoxidase detection strip 77
4.8.1 Working of lactoperoxidase detection strip 78
4.8.1.1 Using TMB liquid substrate for membranes 78
4.8.1.2 Using 4-AAP and Phenol 79
4.9 Validation of paper based strips in milk products (*Khoa, Dahi*) 80
4.9.1 Validation of paper based strips in *Khoa* 80
4.9.1.1 Validation of glucose strips in *Khoa* 81
4.9.1.2 Validation of sucrose strips in *Khoa* 81
4.9.1.3 Validation of maltodextrin strips in *Khoa* 82
4.9.2 Validation of paper based strips in *Dahi* 83
4.9.2.1 Validation of glucose strips in *Dahi* 83
4.9.2.2 Validation of sucrose strips in *Dahi* 83
4.9.2.3 Validation of maltodextrin strips in *Dahi* 84
4.10 Easy handling strip at end point users 85
5.0 SUMMARY AND CONCLUSION 86-87
6.0 BIBLIOGRAPHY i-xvi

LIST OF FIGURES

Figure No.	Title	Page No.
2.1	A typical lateral flow assay strip.	15
3.1	Construction of lateral flow strip.	46
3.2	Dimensions of a lateral flow strip.	48
4.1	Working of neutralizer detection strip (a) in control milk sample, strip color does not change (b) in neutralizer (NaOH at 0.1% level) adulterated milk sample strip colour changes to green.	55
4.2	Effect of neutralization on pH of milk.	57
4.3	Study of limit of detection of neutralizer detection strip with various neutralizers in milk.	58
4.4	Hydrolysis of urea by urease	59
4.5	Working of urea detection strip (a) in control milk sample strip colour does not change (b) in urea (at 0.1% level) added milk sample strip colour changes to deep red.	59
4.6	Study of response time of urea detection strip in milk samples containing urea at two different levels (100 mg/ 100 ml of milk and 120 mg/ 100 ml of milk).	60
4.7	Study of limit of detection of urea detection strip. The numerical values indicate the total urea content (natural + added) in milk in mg/100 ml.	61
4.8	Mechanism for the detection of sucrose in milk.	62
4.9	Working of sucrose detection strip (a) In control milk sample strip color does not change (b) in sucrose (at 0.1% level) adulterated milk sample strip color changes to red.	63
4.10	Study of response time of sucrose detection strip. The study was done in two milk samples containing 0.1% and 0.12% added sucrose. In each pair, the left hand side of the strip indicated control milk sample while right hand side strip indicated milk sample added with sucrose.	63
4.11	Study of limit of detection of sucrose detection strip.	64
4.12	Working of hydrogen peroxide detection strip (a) in control milk sample strip colour does not change (b) in hydrogen peroxide (at 0.01% level) adulterated milk sample strip color changes to brown.	65

4.13	Study of response time of hydrogen peroxide detection strip in milk. (a) pure milk sample (b) Milk adulterated with 0.01% of hydrogen peroxide.	65
4.14	Study of limit of detection of hydrogen peroxide detection strip.	66
4.15	Working of detergent detection test strip. The results were inconclusive and thus further work was abandoned.	67
4.16	Competitive format for Lateral flow assay.	67
4.17	Color changes during preparation of AuNPs.	69
4.18	Absorption spectra of AuNPs prepared in the laboratory by citrate reduction method.	69
4.19	Particle size of AuNPs by dynamic light scattering.	70
4.20	Zeta potential of AuNPs by dynamic light scattering.	70
4.21	Detection of soymilk in borate buffer.	73
4.22	Detection of soymilk in skim milk.	74
4.23	Detection of soymilk in whole milk.	74
4.24	Reaction mechanism of BCIP/NBT red and Alkaline Phosphatase	75
4.25	Reaction of alkaline phosphatase with colouring reagent in liquid and dry phase. (A). Differentiation between raw and pasteurized milk in liquid phase; (B). Differentiation between raw and pasteurized milk using dry reagent chemistry (a_1- raw milk, b_1- pasteurized milk)	76
4.26	Reaction of alkaline phosphatase with colouring reagent in liquid and dry phase. (A). Differentiation between raw and pasteurized milk in liquid phase; (B). Differentiation between raw and pasteurized milk using dry reagent chemistry (a_1- raw milk, b_1- pasteurized milk)	77
4.27	Reaction of lactoperoxidase with colouring reagent in liquid and dry phase. (A). Differentiation between raw and milk heated at 80°C in liquid phase; (B). Differentiation between raw and milk heated at 80°C using dry reagent chemistry (a_1- raw milk, b_1- milk heated at 80°C)	79
4.28	Reaction of lactoperoxidase with colouring reagent in liquid and dry phase. (A). Differentiation between raw and milk heated at 80°C in liquid phase; (B). Differentiation between raw and milk heated at 80°C using dry reagent chemistry (a_1- raw milk, b_1- milk heated at 80°C)	80
4.29	Detection of glucose in khoa. (A). Detection of glucose at 0.5% level; (B). Detection of glucose at 1% level (a_1- pure khoa, b_1 - khoa prepared from milk containing 0.5% glucose, b_2- khoa prepared from milk containing 1% glucose)	81

4.30	Detection of sucrose in khoa. (A). Detection of sucrose at 0.5% level; (B). Detection of sucrose at 1% level (a_1- pure khoa, b_1 - khoa prepared from milk containing 0.5% sucrose, b_2- khoa prepared from milk containing 1% glucose)	82
4.31	Detection of maltodextrin in khoa. (A). Detection of maltodextrin at 0.5% level; (B). Detection of maltodextrin at 1% level (a_1- pure khoa, b_1 - khoa prepared from milk containing 0.5% maltodextrin, b_2- khoa prepared from milk containing 1% maltodextrin)	82
4.32	Detection of glucose in Dahi. (A). Detection of glucose at 0.5% level; (B). Detection of glucose at 1% level (a_1- pure Dahi, b_1 - Dahi prepared from milk containing 0.5% glucose, b_2- Dahi prepared from milk containing 1% glucose)	83
4.33	Detection of sucrose in Dahi. (A). Detection of sucrose at 0.5% level; (B). Detection of sucrose at 1% level (a_1- pure Dahi, b_1 - Dahi prepared from milk containing 0.5% sucrose, b_2- Dahi prepared from milk containing 1% sucrose)	84
4.34	Detection of maltodextrin in Dahi. (A). Detection of maltodextrin at 0.5% level; (B). Detection of maltodextrin at 1% level (a_1- pure Dahi, b_1 - Dahi prepared from milk containing 0.5% maltodextrin, b_2- Dahi prepared from milk containing 1% maltodextrin)	84
4.35	Steps followed for making strips easy to handle.	85

LIST OF TABLES

Table No.	Title	Page No.
2.1	Colour-scale of urea detection strip developed by Kumar *et al.* at different level of urea in milk	7
2.2	Advantages of strip based methods over wet chemical methods	33
3.1	Solutions having different concentrations of A and B	42
4.1	pH of milk containing individual neutralizers (NaOH, Na_2CO_3 and $NaHCO_3$) at different levels.	56

LIST OF ABBREVIATIONS

4-AAP : 4-amino antipyrine
AAS : Atomic absorption spectroscopy
ABS : Alkyl benzene sulphonic acid
ALP : Alkaline phosphatase
AOAC : Association of Official Analytical Chemists
ARS : Agricultural Research Service
BIS : Bureau of Indian Standards
CHE : Cholinesterase
COB : Clot on boiling
DAM : Diacetyl monoxime
DCP : 2,4-dichlorophenol
DGHS : Directorate General of Health Services
DMAB : p-dimethyl amino benzaldehyde
DNA : Deoxyribo nucleic acid
EDTA : Ethylene diamine tetra acetic acid
FIA : Flow injection analysis
FSSAI : Food Safety and Standards Authority of India
FSSR : Food Safety and Standards Regulations
FT-IR : Fourier transformed infrared
GOD : Glucose Oxidase
HRP : Horse radish peroxidase
IDF : International Dairy Federation
ISO : International Organization for Standardization
LOD : Limit of detection
LFA : Lateral flow assay
NAD : Nicotinamide adenine dinucleotide
NADH : Nicotinamide Dinucleotide Dehydrogenase
NDRI : National Dairy Research Institute
SFA : Segment flow analysis
SNF : Solid not fat
TBHBA : 2,4,6-tribromo 3-hydroxy benzoic acid
TCA : Trichloroacetic acid
TMB : 3,3’5,5’-tetramethyl benzidine

CHAPTER – 1

Introduction

1. INTRODUCTION

Adulteration of food products continues to represent a major concern for consumers. Adulteration of food misleads the consumers and can pose serious risks to health. Among the various food products, milk is perhaps the most widely adulterated food item. This has also been highlighted in a recent survey (January 2012) done by Food Safety and Standard Authority of India (FSSAI) and by various media reports. As milk is widely consumed by every section of society, the adulteration of milk and milk products is a major concern for law enforcement agencies. The lack of timely action against the adulterators by the enforcement agencies and lack of easier and rapid methods for detection of adulteration further encourages this menace. Consumers are not aware of the methods and many of the chemicals used for the detection of adulteration in milk are of hazardous nature. Sucrose, urea, neutralizers, salt, detergents, hydrogen peroxide were present in 22%, 60%, 26%, 82%, 44% and 32% milk samples respectively obtained in Hyderabad (Singuluri and Sukumaran, 2014). Different studies carried out in our country indicate adulteration of milk products also. In Northern Indian states *paneer* and *khoa* were reported to be adulterated with glucose, neutralizer and starch (Sharma *et al*., 2005*)*. In a study conducted in Coimbatore revealed that the extent of adulteration in buffalo milk was found to be 36.74%, and that of cow's milk was 29.27%. The extent of adulteration of milk in general was found to be 33.33 %. During the analysis of food items carried out in Hisar district of Haryana revealed that maximum adulteration was found in milk products (34.07%) followed by cereals and their products (19.77%), spices and condiments (18.68%), pulses (14.29%) and miscellaneous food items (13.19%). In another study, different food items were analyzed in Uttar Pradesh for adulteration, after two surveys, it was found that milk was the most frequently adulterated consumable. About 33.8% of samples in first survey and 37% of milk samples in the second survey were found adulterated. Besides 14 –16% of *butter* and *ghee* were also found adulterated at the end of the surveys. In another survey conducted across Delhi by the Prevention of Food Adulteration Department and six samples of *vanaspati ghee* were found to be of substandard quality. Moreover, in several samples *palm oil* was being sold as *vanaspati ghee*

(Tiwari *et al.,* 2013). About 43% of *khoa* samples were found adulterated with non milk fats (Kala, 2012) in Karnataka.

To overcome the menace of adulteration of milk, consumer friendly rapid methods for detection of adulteration of milk are required. Efforts have been made in the past for developing rapid tests for the detection of various adulterants in milk. Simple methods have been developed for the detection of common adulterants by NDRI, in the form of 'easy to carry kit'. In addition there are many private companies which are marketing milk adulteration detection kits. Bureau of Indian Standards (BIS, 1960) has also prescribed rapid methods for detection of adulteration of milk. The existing milk adulteration detection methods are based on the wet chemistry and include basic experimental techniques like measuring, mixing, weighing and boiling of chemicals. So it requires skilled personnel to prepare the reagents. In addition, it requires number of glassware as each single test requires a separate sample test tube and milk/reagent dispensing system. Though, these methods work well and have been validated in the milk system, still development is required in the area of rapidity, sensitivity, cost and accurate detection tests of the adulterants as a routine analysis in the dairy plants.

Dry reagent based strip tests are an attractive alternative to conventional wet chemical methods having the advantage of long stability and chemicals are less likely to be wasted. It also include lower costs, ease in handling, short analysis time and the ability to make quick decisions without the use of a conventional laboratory. In the dry chemistry strip based methods, paper strips are impregnated with dry reagents to which the milk sample is added. The dry chemistry technology is widely applied in clinical and food analysis by using classical organic reagents, enzymes and antibodies. Dry reagent test strips have suitability to be used as an alternative for emergency tests and do not require skilled personnel as the results can be interpreted from the color change with naked eye. Thus development of such test strips can be proved as best way to check the adulteration of milk at the reception docks in dairy plants, at farm level as well as in households.

Earlier efforts have been made in our laboratory to develop test strips for the detection of various adulterants such as neutralizers, urea, glucose, hydrogen peroxide and maltodextrin (Panchal, 2013; Kumar, 2014; Sharma *et al*., 2016a; Sharma *et al*., 2016b). In this study efforts will be made for development of new strips on some selected adulterants (neutralizers, added urea, hydrogen peroxide, sucrose and detergent) and validation of the strips (glucose, sucrose and maltodextrin) in some selected dairy products (*dahi, khoa*) along with the development of a rapid lateral flow assay for the detection of soymilk in milk. Efforts would be made to increase the sensitivity and working of already developed adulteration detection strip. Also attempt would be made to develop new paper based strips for detection of indigenous enzymes (alkaline phosphatase and lactoperoxidase) in milk with a view to assess heat treatment history of milk.

Therefore, the present research work is contemplated with the following objectives:

1. **To develop paper based strips for detection of selected adulterants in milk.**
2. **To develop paper based strips for detection of presence of selected indigenous enzymes in processed milk.**
3. **To validate the strip based tests for detection of adulterants in selected milk products.**

CHAPTER – 2

Review of Literature

2. REVIEW OF LITERATURE

2.1 Dry reagent chemistry

2.1.1 Introduction

Dry chemistry refers to the use of strips impregnated with dry reagents to which the specimen/test sample is added. It is also synonymously named as ‘dry phase chemistry’ or ‘solid phase chemistry’. According to Shepherd (1986) “dry reagent chemistry refers to the reagent format with unitized reagents with a ready to use, convenient instrument system to provide qualitative and/or quantitative results”. Common features of dry phase chemistry are minimum sample preparation and long shelf life before first use (some systems require refrigerated or frozen reagent storage). The technology of dry chemistry has been used widely in clinical chemistry but is also well accepted in other branches of analytical chemistry including food science. Dry reagent chemical tests, such as indicator papers, dipsticks or tableted reagent systems, in research terms, are the Cinderella of the sensor world. In their simplest form, all that is required is a dip and measure test, performed by human observers who will make some sort of decision (often just a Yes/No choice) on the basis of the result. In other formats, these tests are used with some form of measurement instrument, typically a Reflectance Spectrometer. Consequently, dry reagent chemical tests have much to offer not merely for the analyst but also for the public at large. When anyone can measure the nitrate concentration of their tap water or the sodium content of their food, those responsible for the quality of these products will become ever more accountable to the consumers.

2.1.2 Past and current activity

The use of dry phase chemistry can be traced back over 2000 years. Ancient Greeks developed a papyrus test to detect iron adulteration of copper sulphate (Edomonds *et al*., 1997). In the nineteenth century a filter paper impregnated with sodium carbonate was used as a test for uric acid (Diebold *et al.,* 1991). The use of fast simple tests in the form of Reagent Strips for qualitative analysis in clinical laboratory tests also dates back to the 19th

Century. A classical example is the litmus paper used for testing the alkalinity of a solution (Walter, 1983). The litmus paper principle formed an early basis for other solid phase chemistries, such as the analysis of glucose and reducing sugars.

The first qualitative analysis of glucose in urine using solid phase chemistry reagents was described by Maumene (1850). A technique that combined the reagent and the sample on a filter paper was developed by Feigl (1935). The chemical reaction takes place and the developed color is proportional to the concentration of the analyte under investigation. Enzymatic tests for glucose in urine were first developed by Free (1957) and Comer (1956), afterwards other parameters became accessible e.g. pH (Free and Free, 1975), occult blood (Leonards, 1962), bilirubin (Free and Free, 1953), urinary ketone bodies (Free and Free, 1971), protein (Compton, 1945) and others. A single tablet method for the determination of glucose in urine was patented (Free and Free, 1971). Many of these tests and their potential as possible self-testing methods were reviewed by Free & Free (1984).

Gradually the tests evolved to semi-quantitative estimations using color scales and dual color blocks (Marks *et al.,* 1965; Tritschler *et al.*, 1979). Dextrostix (Ames) Reagent Strips were introduced in 1964 as a first rapid disposable test for blood sugar. In 1969 this system was further refined using a reflectance reading Instrument (Mazzaferri *et al.,* 1970; Kattermann *et al.,* 1974; Standl *et al.,* 1973). In the 1970's more sophisticated solid phase chemistry systems emerged for quantitative analyses of blood constituents; this was followed by the development of an adapted Instrumentation. Several licenses were taken not only for the development of reflectometers reading strips but also for tablets (Ger Offen patent DE. 3,020568, US patent 4, 373, 318, JP 5821, 145 and Ger Offen. DE 3,112,031). In the subsequent years, there has also been an increase in the development of dip sticks for quantification of specific gravity (Burkhardt *et al.,* 1982; Luft *et al.,* 1984; Kaehler and Weiland, 1983) and urinary chloride and sodium concentrations without further instrumentation. The application of the multilayer film technique, first developed by the Kodak Company (Curme *et al.,* 1978; Spayd *et al.,* 1978) gave a new impulse to the development of solid phase chemistry reagents.

A literature survey yielded some papers covering the chemistry, technology or use of dipstick or dry reagent chemistry systems in predominantly clinical and food analysis. In one of these papers, ‘competition from developments in dipsticks’ was cited as one reason for the absence of ‘the expected revolution of medical diagnostics by biosensors’ (Edmonds, 1997). Many of these papers deal with devices in which immunoassay systems were used to selectively detect the target analyte; quantification was occasionally performed visually, but more often reflectance or other optical instrumentation was used. It was argued that these latter methods constitute a reagent delivery system rather than a true dry reagent phase test. The presence of a sophisticated and relatively expensive measuring instrument certainly precludes a classification as a chemical sensor, as does the further addition of liquid reagents. However, the ease with which the analysis was performed, particularly complete or partial removal of the need to measure out and mix a number of separate reagents, makes this dry reagent delivery approach an attractive alternative to conventional wet chemical methods.

Correlation between results obtained using dipstick or dry reagent methods and the more conventional wet reagent methods was found to be good for analyses as diverse as the determination of salivary digoxin (Miles *et al.,* 1992) or the determination of potassium in plasma and serum (Ng *et al.,* 1992). The detection of myoglobinuria in urine provides a good example of a procedure in which the dipstick is the primary analytical tool (Robitaille *et al.,* 1995).

Dry reagent chemistry has also been applied in the food quality analysis and analyte quantification. An enzymatic method for the detection of glucose in milk was described by Mal *et al*. (1988). The reaction utilized the enzyme glucose oxidase to catalyze the formation of gluconic acid and hydrogen peroxide from the oxidation of glucose and the second enzyme peroxidase catalyzed the oxidation of potassium iodide present in the ‘diastix strip’ by the hydrogen peroxide to form free iodine. The method was stated to detect minimum 0.05% glucose. Pal *et al.* (1989) modified the above mentioned method for the rapid detection of added sucrose in milk. Sucrose was hydrolyzed to glucose and fructose by ‘invertase enzyme’ and the resultant glucose was detected by diastix strip. A visually evaluable dry reagent strip for rapid semi quantitative estimation of urea in adulterated milk was developed by Kumar *et al.* (2000). The method is based on the (i) urease reacting with urea to liberate ammonia and carbon dioxide and (ii) liberated ammonia reacting with a specific chromogen to change color of the strip from light yellow to magenta, which is visible with naked eyes. Color change after 30 s is compared with the color chart to obtain semi quantitative results. The color of the strip changes from yellow to magenta.

Table 2.1. Colour-scale of urea detection strip developed by Kumar *et al.* (2000) at different level of urea in milk.

Sr. No	Urea in milk (g/l)	Colour of the strip
1	0-0.2	Yellow
2	0.2-0.7	Peach
3	0.7-1.2	Reddish brown
4	1.2-1.7	Pink
5	1.7-14	Magenta

Quick, simple and economical test using dry reagent strips for detection of milk pasteurization was developed (Sharma *et al.,* 2003). It is based on alkaline phosphatase (ALP) reaction with *p*-nitrophenyl phosphate in the presence of water to liberate *p*-nitrophenol and inorganic phosphate. *p*-nitrophenol on reacting with a specific chromogen changes the colour of the strip from light blue to green, which is visualized by the naked eyes. The strip has a sensitivity of >0.5 units/L. The strips may be used in dairy industries and remote areas where expensive instruments are not available. The strip has also been claimed to stable for more than a year at room temperature. Narang and Khokhar (2004) invented dry reagent chemistry based spot test for testing urea in milk. The process of testing comprises steps of putting a drop of indicator solution and an equal amount of milk to be tested on a filter paper. The technology of this test has been patented. A quick and simple biostrip technique for detection of galactose in food was developed by immobilizing galactose oxidase, peroxidase and chromogens on to a polymeric support with a working range of 10 to 50 g galactose per litre and 2 min of response time (Sharma *et al.,* 2004). A strip on lactoperoxidase in raw milk was developed by (Che *et al*., 2015) using hydrogen peroxide and tetramethylbenzidine as a chromogen. The minimum LP concentration detected by the test strip was 1-2 mg/L. Hydrogen peroxide detection strips were also used to determine the extent of pasteurization in whole milk (Marks *et al.,* 2001). In this method the presence or the absence of the unutilized hydrogen peroxide is detected using thiocynate and lactoperoxidase. An optical glutamate biosensor test strip based on stacked membranes of nafion/sol–gel and chitosan was fabricated with immobilized enzymes L-glutamate oxidase and horseradish peroxidase, which sensed the presence of L-glutamate with an aid of indicator dye, 3,3,5,5- tetramethylbenzidine. Quantitative analysis was performed by measuring the reflectance intensity of the color change at 550 nm, which gave a linear response range of 0.01–0.30 mM to L-glutamate with a limit of detection of 5 µM. The strips were successfully applied for the estimation of L-glutamate in common food items such as sauces, soups, processed food and flavour enhancers (Muslim *et al.,* 2011).

Due to increasing prevalence of multidrug-resistant human pathogenic bacteria as a cause of excessive use of antibiotics in veterinary medicine and as growth promoters in stock farming, Nils *et al.* (2007) developed a novel generic dipstick-based technology for rapid and precise detection of tetracycline, streptogramin and macrolide antibiotics in food samples. The generic dipstick consists of either nitrocellulose, nylon or polyvinylidenefluoride (PVDF) membrane strips coated with streptavidin and immobilized biotinylated operator DNA, which acts as capture DNA to bind hexa-histidine (His6)-tagged bacterial biosensors. Antibiotics present in specific samples triggered the dose-dependent release of the capture DNA–biosensor interaction, which, after dipping into two different solutions, results in a correlated conversion of a chromogenic substrate by a standard His6-targeted enzyme complex. This can be quantified by comparison of the dipstick to a standardized color scale or by assessing the terminal solution at 450 nm. Increasing public concern about pesticide contamination of food and the environment has increased the demand for broader and stricter pesticide monitoring. As a result of this, a cholinesterase (ChE)-based dipstick-type assay for the class-specific detection of organophosphate (OP) and carbamate (CM) pesticides was developed by Hwa *et al.* (2007).

2.1.3 Lateral Flow Assay

Lateral flow assay was first developed in 1956 by Singer and Plotz. It is also called the immunochromatographic assay, intended to detect the presence (or absence) of a target analyte in sample. Particles able to produce color are conjugated to the specific biological components of the assay, (antigen/antibody/aptamer). The results are interpreted on the reaction matrix or read either by eye or using a reader. Lateral flow (immuno) assays are currently used for qualitative, semi quantitative and to some extent quantitative monitoring in resource-poor or non-laboratory environments. Applications include tests on pathogens, drugs, hormones and metabolites in biomedical, phytosanitary, veterinary, feed/food and environmental settings. We describe principles of current formats, applications, limitations and perspectives for quantitative monitoring. Originally the technique was called “sol particle immunoassay” (SPIA). Lateral flow assays (LFAs), i.e. prefabricated strips of a carrier material containing dry reagents that are activated by applying the fluid sample, are important for diagnostic purposes, e.g. to ascertain pregnancy, failure of internal organs (e.g. heart attack, renal failure or diabetes), infection or contamination with specific pathogens including biowarfare agents, presence of toxic compounds in food, feed or the environment and abuse of (illicit) drugs.

2.1.3.1 Principle of Lateral Flow Assay

The basic principle underlying in LFA is the movement of a liquid sample containing the target analyte along a strip of polymeric material by the action of capillary flow across various reactive zones where molecules have been attached that exert more or less specific interactions with the analytes (Posthuma-Trumpie *et al*., 2009; Anfossi *et al*., 2013). LFA strip consists of surface layer to carry the sample from the sample pad via the conjugate pad along the strip encountering the detection zone up to the absorbent pad. Absorbent pad maintain the proper flow of liquid by absorbing extra fluid. Labeled analyte (colloidal gold, dyed polystyrene, phosphor (Corstjens *et al*., 2003), dye-encapsulating liposomes, quantum dots, colored or fluorescent or paramagnetic monodisperse latex particles or liposomes or colloidal carbon) are dried on conjugate pad and after addition of the sample, this material will interact with the fluid flow. Two lines are sprayed on the nitrocellulose membrane strip: a test line and a control line. At the test line the recognition of the sample analyte and the reporter will result in the required response. Accumulation of labeled conjugate on test line and control line by immunological recognition system generates detectable signals and results are visualized either by eyes or using a lateral flow strip reader. Control line confirms a proper flow of the liquid through the strip (Posthuma-Trumpie *et al.,* 2009).

2.1.4 Structural components of Lateral flow assay strip

2.1.4.1 Membrane material

The membrane used as strip material is probably the single most important part the test system. Most often the strip material is made of nitrocellulose (Oku *et al.,* 2001; Zhu *et al.,* 2002; Vam *et al.,* 2004) available from Whatman, Pierce, Advanced Microdevices (India) or Millipore. Several other polymeric materials are also available, e.g. nylon (Buechler *et al.,* 1992) (Millipore, Pall Biosciences), polyethersulfone (Edwards *et al.,* 2006; Kalogianni *et al.,* 2007) (Pall Biosciences or Nalgene Nunc), polyethylene (Sanchez *et al.,* 2005) (Porex) or fused silica. The capillary forces of the carrier material are not only important, but also the ease of binding and immobilizing proteins necessary for sequential selection, reaction and detection. For nitrocellulose, various pore sizes are available, varying from 0.05 to about 12 µm. However, the pores are not randomly distributed because of the manufacturing process, and a better description of the strip material should be capillary flow time, i.e., the time for the fluid front to travel into the strip material and is expressed as seconds per centimetre. First of all, the pore size and the material are important for the transport of the label (Henderson and Stewart, 2001). A second requirement is the speed with which the complex of the sample analyte and the label are transported through the membrane allowing optimal reaction time. These parameters are also important when the sample has a high viscosity or contains fat globules, e.g. milk. Usually nitrocellulose material to prepare the strips can be stored at ambient temperature and humidity; however, at low humidity, handling of the material can be difficult owing to accumulation of static electricity. For polyethylene, the pore size can be controlled precisely, and when there is need for such properties, i.e. better reproducibility during the assembly and consequently better reproducibility during the tests, using this strip material is an option. It might be noted here that larger pore sizes will widen the test line, and the sensitivity of the test will decrease, because faint lines can be missed. These membranes are classified based on the flow time and flow rate (sec/cm^2); usually higher flow rate provide more sensitivity and vice-versa. If strip's flow is too fast sensitivity decreases, and if flow is slow specificity decreases, so we can choose according to our requirement.

2.1.4.2 Material of the sample pad

The sample pad can be used to perform multiple tasks; the most important is the promotion and even distribution of the sample to the conjugate pad. The sample pad can also be impregnated with, e.g. proteins, detergents, viscosity enhancers and buffer salts to influence the flow rate of the sample. The aim of such additions can be to increase the sample viscosity, to increase the reaction time at the conjugate pad or even to chemically modify the sample for binding at the test line. The pores in the sample pad can be symmetrically, homogeneously or in homogeneously distributed, or can be asymmetrically distributed, providing an initial filter to remove coarse material, e.g. whole cells. Asymmetrical pore size distribution can be obtained from, e.g., Pall Ahlstrom (Cytosep) (Choi *et al.,* 2004) or from Advanced Microdevices (India) (Leung *et al.,* 2003).

2.1.4.3 Material of the absorbent pad

The wick is the engine of the strip. It is also known as absorbent pad or reservoir. Main purpose of this pad is to pull out all of the fluid added to the strip and to hold it for the duration of the assay. It does not allow releasing this fluid back into the assay, if it allows false positives result can occur. When an absorbent pad is used, the amount of sample can be increased to improve sensitivity. Most often used material is typically high-density cellulose.

Numerous suppliers of these materials are available, with much of the supply is coming from Advance Micro device (Ambala, India), Millipore, Whatman (Kent, UK), Ahlstrom (Helsinki, Finland), and Pall Gelman (East Hills, NY, USA) etc,.

2.1.4.4 Conjugate Pad:

It is also known as reagent pad. The role of this pad is to accept the conjugate, hold it stable over its entire shelf life, and release it efficiently and reproducibly when the assay is run. Pre-treatment is done by immersion of the pad in aqueous solutions of proteins, surfactants, and polymers, followed by drying. The choice of labels and conjugation methods are important for conjugation system. The materials of choice are glass fibres, polyesters, or rayon. For best results, the materials must be hydrophilic and allow rapid flow rates. The conjugate pad must not destabilize the conjugate over the entire shelf-life.

2.1.4.5 Labels for Detection:

The particles used as a label for detection are colloidal gold (Leuvering *et al.,* 1980; Oku *et al.,* 2001; Aveyard *et al.,* 2007; Nielsen *et al.,* 2007) colored or fluorescent or paramagnetic mono disperse latex particles (Gussenhoven *et al.,* 1997; Danks and Barker, 2000; Greenwald *et al.,* 2003) or liposomes or colloidal carbon or quantum dots. Most commonly used labels are colloidal gold as it is less toxic, easy to prepare in lab and of low cost. Its color is intense, and no additional development process is needed for visualization. The choices of label are governed by different factors such as if covalent attachment of the protein to the particle is required then only activated latex particles are used because in case of colloidal gold the binding with protein is typically achieved via passive absorption. Colloidal gold is used most often. An important requirement for the nanoparticle labels is their colloidal stability in solution. Among others, this can be achieved upon coating the binding proteins on the surface of the nanoparticle. Electrical charge can be generated by choosing the right conditions (i.e. buffer composition, buffer strength, pH). A stable suspension ensures a uniform distribution of the conjugate when preparing the conjugate pad. The size of the gold nanoparticles can be controlled during manufacturing (Laitinen and Vuento, 1996, Aveyard *et al.,* 2007). Better sensitivity using larger particles was observed; however, the stability of the colloid was diminished with particles over 40 nm (Laitinen and Vuento, 1996). This problem was not mentioned in (Aveyard *et al.,* 2007), where an optimum was found using gold nanoparticles of 80 nm. Liposome colloids can be instable as well, so in a typical application, the liposomes were immobilized and the label ruptured the liposomes, releasing an electro active compound in a concentration-dependent way (Lee *et al.,* 1999).

2.1.4.6 Position of the test line

The position of the test line is important for the performance of the assay. More interaction time is available when the test line is farther downstream from the sample application side of the strip. An optimum is usually obtained, as the speed of movement of the fluid decreases with distance.

2.1.5 Formats for Lateral Flow Assay

Different formats of assays are available; these are chosen depending on the type of analyte to be identified and the ligand used for the recognition. These are designed to confirm the presence or absence of analyte in the sample. When exclusively antibodies are used as recognition elements then the test is called "lateral flow immunoassay (LFIA)". In such case two common formats are in use; competitive (indirect) assays and the sandwich (direct) assay. A combination of antigen-antibody interaction and specific tagged nucleic acids (aptamers) can also be employed. Nucleic acid can be captured on lateral flow as ligand either in antibody dependent or antibody independent manner. Antibody dependent format is called "nucleic acid lateral flow immunoassay (NALFIA)"; it employs an antibody capture line and a labeled amplicon or oligonucleotide probe of complementary sequence to the amplicon (Mens *et al.,* 2012). Alternatively, antibody independent format called "nucleic acid lateral flow assay (NALF)" is also used wherein nucleic acid hybridization is employed as recognition element (Aveyard *et al*., 2007). Aptamer based lateral flow assay for diagnostic applications has also been explored (Liu *et al.,* 2006; Mazumdar *et al.,* 2010).

2.1.5.1 Competitive formats

Competitive formats are typically used when testing for small molecular weight analytes with single antigenic determinants, which cannot bind to two antibodies simultaneously e.g. antibiotics. In this format, a positive result is indicated by the absence of a test line on the reaction matrix i.e. only one line will appear. If the result is negative than both test as well as control line will appear. In this format two layouts are possible. In the first layout design; antibody are sprayed at the test line, a mixture of sample analyte and labeled analyte are applied at the conjugate pad and the sample analyte and labeled analyte compete for binding sites on the antibody at the test line. While in other case analyte-protein conjugate are sprayed at the test line and a mixture of labeled antibody and sample analyte are applied at the conjugate pad, giving the sample analyte a head start for binding to the antibody (O'Keeffe *et al.,* 2003).

2.1.5.2 Sandwich format

Sandwich format are typically used when testing for larger molecular weight analyte with multiple antigenic sites, such as hCG (human chorionic gonadotropin), dengue antigen, human immunodeficiency virus (HIV) etc. In this case, a positive results are indicated by the presence of two lines i.e. test line as well as control line. If test result is negative; then only one line will appear (control line). This assay employs two different antibodies, one specific to analyte (may be raised in rabbit) and other species specific antibodies (may be raised in goat using the above rabbit serum) and they are applied on test line and control line respectively. The conjugate release pad contains a labeled (usually with gold nanoparticles) analyte-specific antibody. Analyte in the sample will bind during the initial chromatographic process to the labeled antibody. The free antigen sites or epitope can bind to the immobilized antibody at the test line. The response is directly proportional to amount of analyte in the sample. The control line containing species specific antibodies will capture the labeled antibody and thus control line will always appear in this format of assay.

2.1.6 Construction of Lateral Flow Strip and Analyte Detection

Different parts of membrane overlap onto one another and are pasted on a plastic backing card using a pressure sensitive adhesive. Then gold nanoparticle conjugated with antibody applied on conjugate pad. Test line is coated with antibody specific to antigen (primary antibody) and affinity purified antibody (secondary antibody) applied over control line. Then the membrane assembly is dried for 1 hr. at 37°C. These membranes are cut into strips of uniform size using cutting instrument. Strips are ready for testing of target analyte in sample. Figure: 2.1 shows a typical lateral flow assay strip

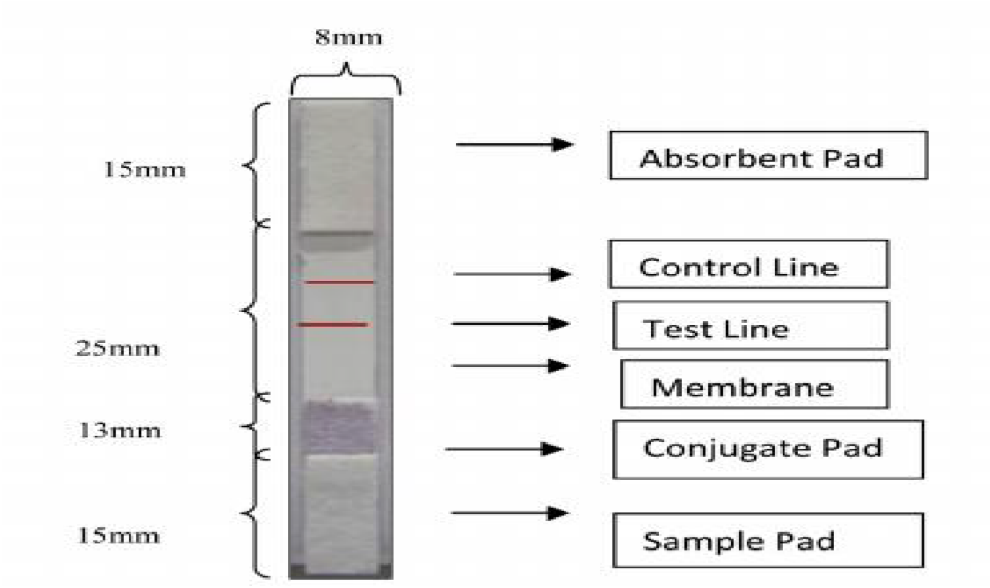


Figure 2.1. A typical lateral flow assay strip

2.1.7 Advantages and Disadvantages of LFA

Lateral flow strips are relatively easy to manufacture, stable and simple to use, can handle small volumes of multiple sample types simultaneously. They have high sensitivity, specificity and good stability. Relatively of low cost, require less time and no extra training is required. It can be integrated with on-board electronics, reader systems and information systems.

Inherent limitation of lateral flow assay includes imprecision that is due to the subjective visual interpretation of test result and to the variability in quality and performance of material from different sources and suppliers. In practice the wetting and spreading of the biological components (analytes) and reporters through the porous membrane is variable and exhibits less than complete coverage. Some portion of sample may not contact the test zone, thus results in a variable loss in signal and diminishes test reproducibility (Volkov *et al*., 2009). Nevertheless, for qualitative and semi-quantitative applications, lateral flow test are often the best choice in term of cost performance.

2.1.8 Quantification using dry reagent tests

In the simplest form tests can be compared with a color chart or color standards of some form. An experienced user (and presumably not one suffering from color-blindness) can obtain truly quantitative results (better than ±10%

accuracy) in certain circumstances. In order to produce a quantitative system, it is necessary to measure the intensity of this color accurately. Unlike wet systems where color intensity is measured with absorption spectroscopy, the measurement technique most suitable for this system is reflectance spectroscopy or occasionally by fluorescence (Zipp, 1900).

a) Reflectance spectroscopy

Two kinds of reflection system are available. The first one is specular reflection, which is the mirror-like reflection from a surface when the angle of the incidence is equal to the angle of the reflection. If the reflecting material is transparent, then some of the incident light will also pass into it and be refracted, that is, bent as it passes through the interface; and the bend angle will be related to the ratio of the indices of refraction of the two media separated by the interface. This type of reflection is of limited value in monitoring solid phase chemistries (Greyson, 1900).

The second one is diffuse reflection, which is a reflection from a matt surface. This is the only reflection of interest for the impregnated fibres technique, while both types are of interest in the multiple layer films (Libeer, 1985).The characteristic feature of a diffuse reflector is that light is reflected from its surface in a hemispherical distribution which is independent of the angle of incidence (Greyson, 1900).

Reflectance from a diffuse reflector is usually given by the ratio of signal coming from the reflecting surface to the incident signal causing the reflection, i.e.

$$\% \mathrm{R} = \frac{\mathrm{I}_{\text{reflected}}}{\mathrm{I}_{\text{incident}}} \times 100$$

For multilayer reflection, the term reflectance density is used. It is a term taken from photographic science-and is defined in terms of the inverse log of reflectance, i.e.

$$\mathrm{D}_{\mathrm{R}} = \log \frac{\mathrm{I}_{\text{incident}}}{\mathrm{I}_{\text{reflected}}}$$

Reflectance measurements are comparable to transmittance measurements in absorption spectrometry. Optical reflection density is linearized through the use of the function developed by Williams and Clapper (1953).

b) Fluorescence spectroscopy

Reactions resulting in the appearance or disappearance of fluorescence are monitored by front face analysis (Kuan *et al.,* 1975) in which irradiation and fluorescence both come from the same surface of the carrier. Unlike reflectance, measured fluorescence is linear with fluorophore concentration in the absence of self-quenching.

2.2 Methods for detection of selected adulterants in milk

2.2.1 Detection of hydrogen peroxide in milk

According to Munday (1957), the Arnold-Mantzel method involving vanadic acid (vanadium pentoxide in 6% sulphuric acid) was the most sensitive method to determine H_2O_2 in milk. This test could detect as low as 80 ppm of H_2O_2 in milk. BIS (BIS, 1961) have specified a method for detection of H_2O_2 in milk using para-phenylenediamine. Milk containing H_2O_2 gives blue color with the reagent under test condition. Barone and Krett (1969) differentiated H_2O_2 treated milk using casein coagulum. The casein coagulum was reacted with sodium nitroprusside. The milk given H_2O_2 treatment of more than 0.02 % gave light orange color whereas untreated milk gave red color of protein precipitate. Gilliland (1969) designed an enzymatic method using horseradish peroxidase and *o*-dianiside as donor. The method could detect H_2O_2 levels of less than 1 µg/ml in milk. Ferrier (1970) improved the method of Gilliland (1969) for faster analysis. He treated the milk with trichloroacetic acid and resultant filtrate was reacted with titanium tetrachloride to give stable color, maxima at 415 nm. The starch iodine test is usually employed to check the residual H_2O_2. A lower limit, which could be detected by this method in milk, was 10µg/ml (Subramanian and Olson, 1968). Detection and determination of hydrogen peroxide by using oxidation indicators comprising 3,3',5,5'-tetraalkylbenzidine. Panchal (2013) designed an enzymatic strip using horseradish peroxidase. A lower limit, which could be detected by this method in milk, was 0.005%.

2.2.2 Detection of neutralizers in milk

Due to tropical environmental conditions in our country and inadequate chilling facilities, the milk gets spoiled in 5-6 h when stored at room temperature. Milk producers and unscrupulous middlemen neutralize the milk to delay the souring or to neutralize the developed acidity. Neutralizers generally used are NaOH, $NaHCO_3$, Na_2CO_3, $Ca(OH)_2$. But addition of neutralizers in milk is prohibited under the FSSR, 2011. This unhealthy and unlawful practice has created the need for suitable methods for detecting these chemicals in milk. Various approaches made in this direction are reviewed here.

2.2.2.1 Colorimetric methods

Colorimetric methods are the simplest methods for the detection of neutralizers. These are based on the color development or color change on the addition of certain chemicals in neutralized milk.

Kosolapov (1940) claimed that as little as 0.05% of sodium bicarbonate could be detected in bulk milk by observing the color of a ring of alcoholic bromothymol blue solution added to the milk. The color of the ring formed was yellow in control (un-neutralized) milk while green in neutralized milk. Winton and Winton (1947) adopted Alizarin Test for detection of neutralizers. About 10 ml of milk was shaken with 0.5 to 1.0 ml of 0.2 % alizarin solution in 90 % ethanol. In the presence of alkali carbonates, the solution turned to a distinct rose color, whereas pure milk became yellow.

Mulay and Ladkani (1973) used Methylene Blue Reduction method to detect sodium bicarbonate in milk. $NaHCO_3$ on reaction with H_2SO_4 liberates CO_2 which causes decrease in oxidation reduction potential of the medium. This results into complete decolourisation of methylene blue in milk and thus indicates the presence of $NaHCO_3$. It was reported that this method would enable the detection of $NaHCO_3$ at 0.4 % and higher levels both in cows and buffaloes milk.

Rosalic acid test, one of the oldest colorimetric methods for detection of neutralizers in milk, is the most popular method for the detection of neutralizers. The method is based on the color change of Rosalic acid in neutralized milk. Owing to the simplicity and ease to detect the color, this method has found a valuable place as a platform test in dairies. Winton and Winton (1947) mentioned this test as Schmidt Rosalic Acid test. The color with neutralized milk was rose red and with pure milk brown-yellow. Again, this test with some modification is listed by Bureau of Indian Standards (BIS, 1961) as Rosalic acid test for carbonates. According to BIS (1961), this test is performed as follows: Five ml of alcohol and a few drops of a 1% (w/v) alcoholic solution of Rosalic acid are added to about 5 ml of milk in a test tube and mixed well. If carbonate is present, a rose-red color appears whereas pure milk shows only a brownish coloration. Further, Arora and Balachandran (1995) reported that in Rosalic acid test a pink colour appeared in the presence of added NaOH, KOH and $Ca(OH)_2$. They recommended that milk should be warmed to 60°C followed by cooling to 30°C before conducting the test. According to them, this test could detect NaOH, KOH and $Ca(OH)_2$ at 15-20 mg/100 ml and Na_2CO_3 and $NaHCO_3$ at 25-30 mg/100 ml of milk. However, they cautioned that the characteristic pink color would appear also due to residual teepol or other alkaline detergents present in glassware and/or milk holding utensils. Kumar (2004) also found that NaOH, Na_2CO_3 and $NaHCO_3$ could be detected at the level of 20, 25 and 40 mg/100 ml of milk, respectively, but that was true only when neutralizers were added to fresh milk in anticipation before the development of acidity. However, in case of milk neutralized with NaOH, Na_2CO_3 and $NaHCO_3$ after extreme souring, the detection level of these neutralizers increased to 55, 105 and 160 mg/100ml, respectively, which were about 3-4 times higher than the corresponding values for fresh milk. Kumar (2004) also observed that the neutralizer could be detected in milk only if the pH of milk was above 6.9, irrespective of the type of neutralizer. Gunnery (1979) had reported earlier that the test results of neutralizer tests were highly influenced by the developed acidity and therefore the sensitivity of the test was dependent on pH of the test sample.

2.2.2.2. Measurement of alkalinity of ash

2.2.2.2.1 Direct titration method for alkalinity of ash

Ashing or incineration of milk destroys the organic compounds leaving the mineral constituents behind. The alkali metal parts in the neutralizers that are added to the milk for neutralization get converted to their oxides or carbonates in presence of the carbon dioxide produced by the decomposition of the organic materials. As a result, the alkalinity of ash of neutralized milk is found to be more than that of normal milk.

Hillig (1941) suggested a method for estimation of the alkalinity of ash for detection of neutralizers in dry skim milk. He reported values for ash alkalinity of un-neutralized dry skim milk ranging from 80 to150, expressed as the ml of 0.1 N HCl required to titrate the ash from 100 g of dry skim milk; the ash alkalinity of neutralized dry skim milk was found to be more.

Winton and Winton (1947) listed two methods for determination of alkalinity of total ash: (i) An indirect method for ash rich in carbonates which consisted of dissolving the ash in an excess of standard 0.1NHCl followed by boiling to remove the carbon dioxide and then cooling and titration of the excess acid with standard 0.1N NaOH using phenolphthalein as indicator. Alkalinity value was expressed as the number of ml of 0.1N HCl per gram of the sample. (ii) A direct method for ash containing little or no carbonate in which about 50 ml water was added to the ash and titrated with standard 0.1N HCl using methyl orange as indicator.BIS (1961) has recommended a procedure based on the alkalinity of ash for detection of neutralizers in milk. Neutralization of milk, whether with lime, soda ash or caustic soda, would invariably increase the ash content and total alkalinity of ash from a fixed quantity of milk. Presence of neutralizer is indicated when the volume of 0.1N HCl required to neutralize 10 ml aqueous solution of ash obtained from 20 ml milk exceeds 1.2 ml.

Patil (1982) observed that the determination of buffer intensity of total ash helped in detecting neutralizers. While addition of carbonates to milk markedly increased the buffering capacity of soluble ash, added sodium hydroxide had no significant effect in both cow and buffalo milk. Thus, determination of buffering capacity of soluble ash helped in establishing the type of neutralizer added to milk. Kumar and Unnikrishnan (2004) found that 60 mg NaOH/100 ml of milk may go undetected by ash alkalinity method.

2.2.2.2.2 Back titration method for alkalinity of ash

Direct method of measurement of alkalinity of ash to detect neutralizer in milk suffers from poor reproducibility due to poor dispersion/solubility of ash in water. To overcome this limitation, Kumar and Unnikrishnan (2004) suggested the dissolution of the ash in an excess of standard 0.1N HCl followed by titration of the excess acid with standard 0.1N NaOH using phenolphthalein as indicator. Earlier this test was described as an indirect method for determination of alkalinity of total ash where methyl orange as well as phenolphthalein (Winton and Winton, 1947) was used as an indicator during titration. Kumar and Unnikrishnan (2004) found excellent reproducibility when phenolphthalein was used as an indicator.

2.2.2.3 Determination of Sodium, Potassium and Calcium

Neutralizers generally used are alkaline substances in the form of carbonates, bicarbonates and hydroxides of sodium, potassium and calcium salts. Therefore, determination of sodium, potassium and calcium content of milk can be a useful parameter for the detection of added neutralizers in milk. Sodium and potassium contents of milk can be conveniently analyzed by FAAS. The sodium, potassium and calcium contents of cow milk varied between 39-64, 121-168 and 104-128 mg/100 g, respectively (Gaucheron, 2005) while the corresponding values in buffalo milk came out to be 47, 112, 188 mg/100 g (Ahmed *et al.,* 2013). Therefore, the increase in sodium, potassium and calcium contents over the natural contents reported for the milk confirms the presence of neutralizers in milk.

2.2.3 Methods for detection and determination of urea in milk

Urea is a small organic water soluble molecule composed of carbon, nitrogen, oxygen, and hydrogen (M.W. 60.06 g/mol) of chemical formula $CO(NH_2)_2$ and is also known as carbamide (Ferguson, 2000 and Jenkins *et al*., 2002). It is a natural constituent of natural body fluids including blood (160-420 mg/l), urine (5000-12000 mg/l) and milk (100-450 mg/l) (Miller, 1971). The amount of urea naturally present in milk is of small quantity (18 - 40 mg/100 ml) compared to that of adulterated milk samples (Sharma *et al*., 2008; Ohlsson *et al.,* 2010).

Urea as an adulterant is used in the preparation of synthetic milk in which it is used as a nitrogen source. In other way it is added in the milk to increase the SNF and heat stability. In such adulterated milk, its concentration is about twenty times more than the normal level (Kumar *et al.,* 2000). Addition of extraneous urea into milk is not permitted and the level of urea in milk should not be more than 700 ppm (FSSR, 2011). From the public health point of view, urea added as an adulterant has a carcinogenic effect on the human body system and can lead to gradual impairment of the body (Dean, 1985 and Fox, 1992). Major problem associated with excess urea happens when kidneys begin to work overtime to rid the body of excess urea, they also excrete large amounts of minerals, the most important of their components is calcium. The high concentration of calcium mixed with uric acid in the kidneys form kidney stones and the body becomes depleted of calcium and draws it out of the bones rendering them weak, the ending result can be osteoporosis (Atukorale, 1979). Excess of urea in milk on boiling decomposes to carbonic acid, acetic acid, and ammonia, those ammonia-containing calculi might be formed by the partial fermentation of urea in the bladder. Also, it may convert into biuret which causes fall in blood pressure and produces strong irritation in the urinary tract (Prout, 2003).

Methods used for detection of urea in milk can be classified into three groups on the basis of their principle involved (Lefier, 1996).

Direct methods: -These are based on the measurement of a coloured complex formed between urea and reagent i.e. colorimetric methods

Indirect methods: - These are based on the measurement of a by-product from urea degradation, which reacts with a reagent – mainly enzymatic method.

Physical methods: - These include infrared measurement and combination of enzymatic method with use of ammonium ion-selective electrodes.

2.2.3.1 Direct methods

In this case, distinction can be made between two procedures which differ according to the reagent used and the experimental conditions.

2.2.3.1.1. DMAB Method

Urea forms a yellow colour complex with *p*-dimethyl amino benzaldehyde (DMAB) reagent in low acidic alcohol solution at room temperature. The intensity of the colour is measured at 440 nm (Lefier, 1996).

$$\text{Urea} + \text{DMAB} \xrightarrow[\text{H}_2\text{SO}_4]{\text{Alcohal}} \text{Yellow Colour Complex}$$

Bector *et al.* (1998) modified this method and found the maximum absorption at 425 nm. According to these workers the colorimetric procedure, using DMAB reagent as a complexing agent is an easy method to determine urea content in milk. Presently this method is widely used in the industry to detect added urea in milk.

2.2.3.1.2 Diacetyl Monoxime (DAM) Method

Urea forms a complex with diacetylmonoxime (DAM) reagent, under hot (90°C), acidic conditions. The chemical reaction is stimulated by ferric ions and is stabilized by thio-semicarbazide (Lefier, 1996). Color intensity is determined spectrophotometrically at 520 nm.

This method can be automated using the segment flow analysis (SFA) method with diacetylmonoxime as reagent. De Jong *et al.* (1992) assessed the SFA method for the determination of urea in milk. The sample does not need to be diluted because the milk proteins are precipitated using trichloroacetic acid (TCA) at 12% final concentration in the manual procedure. They showed that the recovery of added urea averaged 98.5%, with perfect linearity between 5 and 75 mg of urea/100 ml of milk.

2.2.3.2 Indirect methods

These are essentially the enzymatic methods. Urea is hydrolyzed to ammonia and carbon dioxide in the presence of urease enzymes (De Jong *et al.,* 1992).

$$\text{Urea} + \text{H}_2\text{O}_2 \xrightarrow{\text{Urease}} 2\text{NH}_3 + \text{CO}_2$$

In the presence of glutamate-dehydrogenase (GlDH) and reduced nicotinamide adenine dinucleotide (NADH), ammonia reacts with 2-oxyoglutarate to give L-glutamate, whereby NADH is oxidized to NAD+

$$\text{GlDH 2-Oxyglutarate} + \text{NADH} + \text{NH}_3\text{L}^{\cdot} \rightarrow \text{Glutamate} + \text{NAD}^{+} + \text{H}_2\text{0}$$

The amount of NADH oxidized during reaction is stoichiometrically related to the amount of ammonia or to half the amount of urea. The enzymatic determination of urea in milk requires two measurements; one for ammonia content in milk and another for the total NH_3 content after urease is added. The urea content is obtained by subtracting the milk NH_3 content from the total NH_3 content. The enzymatic method is the most wide spread and is used for manual determination and for automated determination methods such as FIA (Flow Injection Analysis) method (Oltner *et al.,*1985a; Anderson *et al.*, 1986) and methods using clinical instrument such as IL 919 glucose creatinine analyser (Oltner and Sjaunja, 1982 b).

2.2.3.3 Physical methods

These include infrared measurement (Nygaard *et al*., 1993) and combination of enzymatic methods with pH or conductivity measurements.

2.2.3.3.1 Infrared measurements

Urea content in milk can be determined by infrared measurement (Nygaard *et al*., 1993). A complete Fourier transformed infrared urea spectrum in Nujol is given in Sigma Library of FT-IR spectra. The urea absorbance spectrum shows four absorption bands at 1690 cm^{-1}, 1600 cm^{-1}, 1472 cm^{-1}, and 1161 cm^{-1}.The spectral bands around 1690 $cm^{-1}$and 1600 cm^{-1} could be attributed to the C=0 stretching vibration of the amide function which absorbs strongly at around 1690 cm^{-1}, and the combination bands of the N-H deformation and C-H stretching vibrations in the primary amides when the substance is in diluted solution. The 1472 cm^{-1} band corresponds to the N-H bending vibrations and 1161cm^{-1} band could be allocated to the -C-N- stretch in the aliphatic amide.

2.2.3.3.2 Enzymatic methods coupled with pH or conductivity measurements

The enzymatic method can also be coupled with pH or conductivity measurements, instead of with the spectroscopic determination of NADH oxidation. In this way, carbon dioxide and ammonia from the urease activity on urea ionize according to the following equilibrium (Lefier, 1996).

$$2NH_3 + CO_2 \longrightarrow 2NH_4 + 2CO^-$$
$$CO_2 + H_2O \longrightarrow H_2CO_3 \longrightarrow HCO_3^- + H^+$$

The first reaction is almost instantaneous while the formation of the HCO^{3-} ion is stimulated by the addition of acetazolamide.

Above mentioned, standard chemical and enzymatic methods are being used in routine manner, but these require lab space, spectrophotometer, trained personnel and are time consuming. To overcome these problems dry chemistry based strip tests were developed. In form of quick, easy and efficient platform test, Kumar *et al.* (2000) developed a visually evaluable dry reagent strip technique for semi quantitative estimation of urea in adulterated milk. The method is based on the (i) urease reacting with urea to liberate ammonia and carbon dioxide and (ii) liberated ammonia reacting with a specific chromogen to change color of the strip from light yellow to magenta, which is visible with naked eyes.

Another method based on dry reagent chemistry was invented by Narang and Khokhar (2004). The method involved steps like putting a drop of indicator solution and an equal amount of milk on a filter paper. The color of the spot formed on a paper indicates positive or negative results. Control samples form yellow colored spot whereas adulterated samples form a red colored spot on a paper. The intensity of red color increases with concentration of urea in milk.

2.2.4 Detection and determination of detergents in milk

Detergents are amphipathic molecules that possess both a hydrophobic (water-fear) and a hydrophilic (water-friend) group that allow them to act as excellent solubilizing agents (Helenius and Simons, 1975). The structure of detergent is key to its ability to function as a solubilisation agent. Detergent molecules contain a polar head group from which extends a long hydrophobic carbon tail (Tanford, 1980). The amphipathic properties of the detergent molecules allow them to exhibit unique properties in aqueous solutions. The polar (hydrophilic) head groups interact with the hydrogen bonds of the water molecules and the hydrophobic tails aggregate resulting in highly organized structures called micelles. Detergents are surface active agents (surfactants) particularly effective in dislodging foreign matter from soiled surfaces and retaining it in suspension. The term usually denotes a synthetic substance that is not prepared by saponifying fats and oils (as is soap).

Detergents are used by unscrupulous people as an ingredient for the preparation of so-called synthetic milk. Detergents are added to emulsify refined oil which is added as a substitute for milk fat.

2.2.4.1 Detection of detergents containing Alkyl Benzene Sulphonic Acid (ABS)

Paradkar *et al.* (2000) reported a method for the detection and estimation of anionic detergent in milk. Methylene blue and chloroform had been used as the detecting reagents. If the methylene blue color extracted from a suspicious sample into the chloroform layer was greater than that extracted from an authentic milk sample, it indicates the presence of detergent in milk qualitatively. For the quantitative estimation of detergents in milk they have recorded the absorbance of the chloroform layer at 653 nm. The absorbance shows the linear relationship (R^2=0.9585) with the increasing detergent concentration. They have also reported some component of cow milk were able to extract methylene from the aqueous layer to the chloroform layer and contribute to the optical density of 0.52, which should be considered as the base value for pure cow milk. Any value than this should be taken to indicate the presence of detergent in milk.

Because of high blank values in chloroform layer (O.D of pure milk sample was 0.52), the method does not work in milk samples containing lower content of detergent and higher O.D values interfered with the interpretation of results by visual observation. Later on Rajput *et al.* (2006) have developed a rapid method for detection of anionic detergent in milk. In this developed method 1 ml of milk is mixed with 1ml of dye solution and then 2 ml of chloroform is added to a test tube (15 ml). The contents were then vortexed for about 15 seconds and centrifuged at about 1100 rpm for 3 min. Intensities of blue color of lower and upper layer are noted. Relatively, more intense blue color in lower layer indicates presence of detergent in milk. On the other hand, relatively more intense blue color in upper layer indicates absence of detergent in milk. The method has been adopted by Food Safety Standards Authority of India (FSSAI) in 2016 for detection of detergent in milk (FSSAI, 2016).

DGHS (2005) have described a method for the detection of detergents in milk which is also based on the principle that a solution of anionic detergent containing added methylene blue when shaken with chloroform dissolves the methylene blue salt of the detergent. The mixture is then treated with a cationic active reagent, which after it has combined with all the free anionic detergent, begins to displace methylene blue from the salt. The end point is taken when sufficient methylene blue has been displaced into the aqueous layer to produce phases of equal color intensity. As the reaction is not stoichiometric, it is essential to carry out standardization using a known cationic detergent similar in nature to the unknown.

In this test, 10 ml of milk sample containing approximately 100- 168 mg of active detergent powder is mixed with 25 ml of methylene blue (0.005 %) and 15 ml of chloroform. The contents are mixed well in a separating funnel. If the chloroform layer is blue or greenish blue, the test for detergent containing ABS is positive. Further confirmation is done by titrating with 0.004 M sodium lauryl sulphate (SLS) solution. On titration SLS reacts firstly with anionic detergent, then displaces methylene blue from the salt. The end point is taken when sufficient methylene blue has been displaced into aqueous layer phase resembling both the phase equal in color intensity.

Barui (2011) has described a new qualitative test for the detection of anionic detergent in milk. In this test, 400 µl milk is required to be added into the detecting reagents (400 µl dye + 800 µl chloroform) and then mixing them by inverting the tubes 20 times. Appearance of purple color in lower layer indicates pure milk whereas blue color indicates presence of anionic detergent in milk. The method can detect 0.015 % of labolene (commercial detergent) or 0.0015 % of sodium dodecyl benzene sulphonate in milk. The method is rapid, not prone to interference by commonly used preservatives and other known adulterants added to milk. Based on this dye-detergent complex extraction method, a quantitative spectrophotometric method has also been developed by measuring the absorbance of the complex at 635nm (Barui, 2011).

2.2.4.2 Detection of detergents in milk using paper chromatography

A paper chromatography method for detection of detergent in milk is described by Barui *et al*. (2011). There is a difference in mobility of methylene blue – detergent complex to that from methylene blue when chloroform was used as mobile phase. The results are available in 10 min.

2.2.4.3 Detection of detergents in milk using bromo cresol purple indicator

This test is based on the principle that milk sample containing detergent gives violet color with bromo cresol purple solution. In the test, 0.1ml of bromo cresol purple solution (0.5 %) is added to 5ml of milk in a test tube, appearance of violet colour indicates the presence of detergent in milk (Srivastava, 2010).

2.2.5 Detection of sucrose in milk

2.2.5.1 Modified Seliwanoff's method

Fructose in cane sugar reacts with resorcinol in HCl to give red color. Fructose can be determined quantitatively by using Carrez 1 & 2 solution. *(Ref:- IS 1479 (Part 1) 1961 (Reaffirmed 2003) Methods of test for dairy industry – Rapid examination of milk, BIS New Delhi).*

2.2.5.2 By high performance liquid chromatography (HPLC)

HPLC–RI can used for determining sugars (glucose, fructose and sucrose) in apple juice (Garza *et al*., 1996), disaccharides in whey permeate (lactose, galactose and lactulose)(Ming *et al*., 1996), oligosaccharides (fructose, glucose, sucrose, maltose and lactose) in plain cereals, sugar coated cereals, canned fruits, canned vegetables, crackers cookies (Casterline *et al*., 1999). HPLC–RI has also been used for determining sugars (sucrose, glucose and fructose), in fruit and drink samples (Yuan and Chen, 1999), sugars in meat products (Vendrell *et al.,* 2000), oligosaccharides in lactose–sucrose systems for determining sucrose inversion by invertase (Kouassi and Roos, 2000) and in sugar casein systems (Brands *et al*., 2001). HPLC–RI appears to be an economical, simple and fast method for determination of sugars.

2.2.6 Detection of Alkaline Phosphatase in milk

Any phosphatase present in the milk splits the substrate, *p*-nitrophenylphosphate, to give *p*-nitrophenol, which is highly colored in alkaline solution. Alkaline phosphatase being an indigenous enzyme in milk is used as a marker for pasteurization as it is inactivated at a temperature just above the destruction of the most heat resistant pathogen found in milk. So its detection helps in finalizing whether milk is pasteurized or not. In presence of *p*-nitro phenyl phosphate, alkaline phosphatase forms yellow colored compound i.e. *p*-nitro phenol in alkaline medium. The test is considered to be satisfied by milk which gives a reading of 10 pg or less of *p*-nitrophenol/ml of milk. Properly pasteurized milk will give no discernible color (Official Methods of the AOAC, 1984).

2.2.7 Detection of Lactoperoxidase in milk

Peroxidases catalyze oxidation of various hydrogen donors at the expense of peroxide molecules (ROOH), such as hydrogen peroxide, hydroperoxide and peracid. Bovine milk contains about 30 µg/ ml of lactoperoxidase (Reiter *et al.,* 1985). It is possible to preserve raw, uncooled milk collected under difficult conditions at ambient temperatures of up to 38°C, and increasing the keeping quality of the milk using lactoperoxidase. Lactoperoxidase activity is relatively stable to heat treatments up to 75°C, but significant decreases in activity occurs when milk is treated at higher temperatures. In general, only traces of enzyme activity remain after holding milk at 82°C, for several seconds In this case, lactoperoxidase is a suitable indicator for controlling the heat load of pasteurized milk. Griffith (1986) suggested that the determination of lactoperoxidase activity could offer a means of determining high pasteurized treatments in milk with temperatures higher than 78°C15 seconds. Milk samples with negative lactoperoxidase activity would indicate temperature / time treatments in excess of this value. (Sharma and Rajput, 2014) developed a rapid test to differentiate between high heated and raw milk by accessing lactoperoxidase activity. The raw (unheated) milk developed pink color under the test conditions, while heated milk (80 °C, 15 s) remained either white or gives pink color with significantly reduced intensity. It is recommended to always use raw milk as positive control for the enzyme assay.

2.2.7.1 Qualitative enzyme assay

Several assays have been used for measurements of peroxidase concentrations. Most assays are based on the principle that the electron donors in oxidized form absorb visible light and can be determined by a spectrophotometric assay. However, it is difficult to compare reported results since there are no standardized peroxidase units upon which such comparisons may be based. Examples of such substances that have been utilized in lactoperoxidase assays are pyrogallol, O-dianisidine and *p*-phenylendiamine (Storch reaction). Guaiacol is used most frequently as the electron donor for peroxidase assay because it gives a colored product. This colored product is not a primary product of the reaction, but a mixture of several final reaction products (Dupouy reaction). When the peroxidase reaction is used for H_20_2 micro assay and enzyme immunoassay, guaiacol is replaced by 2.2'-azino-di-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), 3.3',5,5'-tetramethylbenzidine dihydro chloride and phenols + aminopyrine which give stable and intense colored products. Scopoletin is a good substrate for measuring the activity of lactoperoxidase, because its oxidation product fluoresces. When certain organo - fluoro compounds are used, the fluoride is released during the reaction and the activity can be measured by a fluoride ion – sensitive electrode.

2.2.7.2 Spectrophotometric method

Method

As described by Shindler *et al.* (1976), 25 µl of the sample is added to 5.0 ml of substrate solution (1 mM ABTS in 0.1 M acetate buffer pH 4.4) and mixed thoroughly. A 2.5 ml portion is transferred to each of two cuvettes, which are put in the spectrophotometer. The experiment is controlled that a stable and straight baseline is obtained. 50 µl 5mM H_2O_2 solution, prepared fresh, is added to the sample cuvette and mixed carefully. The increase in absorbance is then measured for 3 to 5 min at 413 nm. The increase in absorbance/min (AA4i3/min) is calculated drawing the tangent to the initial activity.

2.2.8 Detection of Soya protein in milk

Soymilk is liquid extract from soya bean grains with color and composition similar to dairy milk. Due to this reason it is used for adulteration of milk. The production cost of this milk is 60-70% less than that of normal milk and its production is easy. Soymilk is admixed to bovine milk either to sale as fluid milk or to prepare skim milk powder for cheese revenue maximization (Seker and Harper, 2004; Arvanitoyannis and Tzouros, 2005). Soya bean protein is cheaper than bovine proteins and thus presents a lucrative inducement to manufactures to use soy protein in infant formulae and milk replacers (May *et al*., 1982; Dawson *et al*., 1988). In order to prevent it a number of methods have been employed for its detection.

2.2.8.1 Polarimetric method

Carbohydrates of soymilk are different than bovine milk. It contains sucrose, raffinose, stachyose (Wang *et al*., 2003). These sugars are absent in milk and are optically active, so this property of these sugars is used for detection of soya in milk. The protein free filtrate is prepared using mercuric iodide followed by centrifugation and filtration. The protein free filtrate is analyzed by polarimeter. Milk adulterated with 10% soy milk can be detected by this method (Sharma *et al*., 2010)

2.2.8.2 Isoelectric precipitation method

Many of soya proteins have iso electric pH at 5.7 and this property is used to detect adulteration of milk with soya. The major soya proteins (7S and 11S) can be precipitated in the pH range 5.4-6.0 (Liu, 1997). In this pH range major milk proteins are soluble. Coagulum was observed when milk adulterated with soya milk was incubated. This method was capable of detecting presence of soymilk in milk at 5-10% level (Sharma *et al*., 2010).

2.2.8.3 SDS-PAGE method

In this method the identification of soya protein bands in adulterated bovine milk with soymilk. Proteins in soymilk and cow milk are of plant origin respectively, and thus differ in molecular weights. When SDS-PAGE is carried out, four bands specific to soymilk were identified which were clearly identified from bovine milk proteins in adulterated milk. The results of SDS-PAGE method are available within 8 h. This method was capable of detecting presence of soymilk in milk at 5% level (Sharma *et al*., 2010).

2.2.8.4 Immunodiffusion method

This method involves detection of adulteration of milk with soymilk on the basis of interaction of soy specific antisera with soya proteins present in adulterated milk. Immunodiffusion can be carried as per the method described by Johnstone and Thorpe (1996). This method was capable of detecting presence of soymilk in milk at 1% level and giving results in 12 h (Sharma *et al*., 2010).

2.2.8.5 HPLC based method

Since soymilk differ in carbohydrate profile than that of bovine milk so HPLC profile can be used to detect the adulteration. A high-performance liquid chromatography method for the detection of adulteration of milk with soymilk, based on separation of sugars on NH_2 column and their detection by refractive index detector. Sugars were extracted with 20% acetonitrile in the presence of carrez solutions and quantified. The presence of stachyose in milk sample has been used as the marker sugar for the presence of soymilk in milk samples (Sharma *et al*., 2009).

2.3 Conclusion

From the above, it can be stated that dry chemistry and paper strips are the promising alternative for detection of adulteration in milk. These methods have the advantage tabulated as below and would be more acceptable for their use at field level vis-a-vis wet chemistry based methods. The review of literature suggested that dry chemistry based methods have not been exploited fully for their use in milk, particularly for detection of adulterants in milk. Although, methods based on wet chemistry are sensitive enough to detect adulteration in milk, the dry chemistry based method would be more attractive alternative to end users. In this research project, attempt will be made to develop strip based test for detection of some selected adulterants and presence of indigenous enzymes in milk using the principles of dry chemistry. Also the already developed strips will be validated in selected milk products (*dahi, khoa*) to detect the presence of maltodextrin, sucrose, glucose.

Since all the standard methods available for detection and determination of adulterants like urea, neutralizers, sucrose, hydrogen peroxide, detergent, soya protein and enzymes like alkaline phosphatase, lactoperoxidase in milk and milk products require glassware and a laboratory environment to perform, there is need of field level tests. In the past, a few strip based tests have been developed in our lab (Panchal, 2013; Sharma *et al*., 2013 and Kumar, 2014) to detect common adulterants in milk, there is still a scope to improve the performance of such tests.

Table 2.2: Advantages of strip based methods over wet chemical methods

Particular	Strip based method	Wet chemical method
Handling	Easy to use	Handling of reagents, glasswares, etc.
Stability	Stable up to 6 months or more	Require fresh preparation of reagents
Cost	Economical	Comparatively costly
Response	Quick results	Takes time
Technical know how	Not required	Require trained person to perform tests
Volume of sample	Very less (50-100 µl)	Few milliliters
Sensitivity	Very sensitive	Comparatively less sensitive

CHAPTER – 3

Materials and Methods

3. MATERIALS AND METHODS

3.1 Milk samples and other materials

Authentic pooled samples of raw cow and buffalo milk were collected from the Cattle Yard of the Institute and used for all experiments.

3.2 Qualitative detection of added neutralizers in milk using dry reagent strip

3.2.1 Chemicals and materials

1. pH sensitive chromogen.
2. Sodium bicarbonate ($NaHCO_3$)- S6297 (AR grade, Sigma Aldrich, USA)
3. Sodium carbonate (Na_2CO_3)- 13418 (AR grade, Sigma Aldrich, USA)
4. Sodium hydroxide (NaOH)- V800383 (AR grade, Sigma Aldrich, USA)
5. Paper cutter (Deli 8051)
6. pH meter (Thermo Orion 420)
7. Whatman filter paper (No.1) (GE healthcare, UK Ltd.)

3.2.2 Method of preparation of neutralizer detection strip

3.2.2.1 Adsorption of chromogen on a filter paper

Ten milliliters of pH sensitive chromogen solution was taken in clean dry petriplate. A piece of filter paper (6×4 cm^2 size) was dipped in it for at least 5 min. After that, paper was taken out and kept for air drying (2 h) in a BOD incubator at 30°C. After drying, the test paper was cut into small strips of 1×6 cm^2 size, the cut strips were pasted on an inert paper (7 x 6 cm^2) using a non-reactive adhesive. The inert paper strip along with pasted detection strip was then cut into 0.5×6 cm^2 size and sealed in a plastic bottle and stored at room temperature (20-30°C).

3.2.3 Proof of principle of working of prepared dry reagent test strip in neutralizer added milk samples

The milk sample was taken in a 100 ml beaker and brought to room temperature. Appropriate amount of NaOH was added to milk to a final concentration of 0.1%. Each neutralized milk sample was then tested with prepared detection test strip as well as conventional wet chemistry based detection method and compared for colour contrast with that of control milk sample.

3.2.4 Determination of response time

Milk sample neutralized with NaOH at 0.1% level was subjected to determine the response time of the strip. Sample was tested with strip and observed for the time at which optimum colour contrast with that of control sample obtained.

3.2.5 Determination of limit of detection of various neutralizers in milk using test strip

To determine limit of detection of prepared dry reagent strip, the milk sample was neutralized with NaOH, Na_2CO_3 and $NaHCO_3$ at 0.01, 0.03, 0.05 and 0.07% levels for NaOH, 0.01, 0.03, 0.05, 0.07 and 0.09% levels for Na_2CO_3, 0.01, 0.03, 0.05, 0.07, 0.09 and 0.1% levels for $NaHCO_3$. Each milk sample was then tested with test strip and observed for the colour change with that of control milk sample.

3.3 Qualitative detection of added urea in milk using dry reagent strip

The extraneously added urea in milk was detected by the combination of three chromogen reagents which were immobilized on Whatman Paper No. 1.

3.3.1 Chemicals and materials

1. Chromogen 1
2. Chromogen 2
3. Chromogen 3
4. Disodium hydrogen orthophosphate dehydrate-15825 ($Na_2HPO_{4.}2H_2O$) (Qualigens Fine Chemicals, Mumbai, India)
5. Sodium dihydrogen orthophosphate dehydrate-14105 ($NaH_2PO_4.2H_2O$) (Qualigens Fine Chemicals, Mumbai, India)
6. Urea-20885 (LR grade, Qualigens Fine Chemicals, Mumbai, India)
7. Whatman filter paper (No. 1) (GE healthcare, UK Ltd.)

3.3.2 Reagent

1. Phosphate buffer (0.1 M, pH 6.5): Dissolve 1.039 g sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_2O$) and 0.594 g disodium hydrogen orthophosphate dihydrate ($Na_2HPO_{4.}2H_2O$) in 100 ml of distilled water.

3.3.3 Method of preparation of urea detection strip

3.3.3.1 Adsorption of chromogen 1 and 2 on the filter paper

Ten milligram of chromogen 1 and 250 mg of chromogen 2 were dissolved in 10 ml phosphate buffer (0.1 M, pH 6.5) at room temperature. The content was mixed to form homogeneous solution and then solution was poured into a clean dry petriplate. A piece of filter paper (6x4 cm^2 size) was dipped in it for at least 4 min. After that the paper was taken out and kept for air drying (3 hours) in BOD incubator at 30°C.

3.3.3.2 Adsorption of chromogen 3 on the filter paper pre-adsorbed with chromogen 1 and 2

The paper pre-adsorbed with two chromogen solutions was soaked in chromogen 3 solution for at least 5 min and then kept for air drying (2 h) in BOD incubator at 30°C. After complete drying, the test paper was cut into small strips of 1x6 cm^2 size, the cut strips were pasted on an inert paper (7 x 6 cm^2) using a non-reactive adhesive. The inert paper strip along with pasted detection strip was then cut into 0.5×6 cm^2 size and sealed in a plastic bottle and stored at room temperature (4-5°C).using a non-reactive adhesive. It was then cut into 0.7×0.7 cm^2 size and sealed in a plastic bottle and stored at refrigeration temperature (4-5°C).

3.3.4 Proof of principle of working of prepared dry reagent test strip in urea added milk

The milk sample was taken in a 100 ml beaker and brought to room temperature. Appropriate amount of urea was added to milk sample to reach extraneously added urea level of 0.1%. Both the control and adulterated milk samples were tested with developed test strip. For this, each piece of test strip was dipped into milk sample for 5 s, extra milk loaded on the strip was wiped by the side of the beaker and kept aside on a dry non-absorbent surface. The appearance of colour on the strip was observed for positive or negative results.

3.3.5 Determination of response time

Two milk samples added with urea at 0.1 and 0.2% level were subjected to determine the response time of the strip. Each sample was tested with strip and observed for the optimum colour contrast with that of control sample.

3.3.6 Determination of limit of detection of urea in milk using prepared test strip

To determine the limit of detection of developed dry reagent strip, the appropriate level of urea was added to milk so as to achieve total urea content (natural + extraneously added) at 0.04, 0.06, 0.08, 0.1 and 0.12 % levels.

3.4 Qualitative detection of added sucrose in milk using dry reagent strip

3.4.1 Chemicals and materials

1. Chromogen 1
2. Chromogen 2
3. Citric acid ($C_6H_8O_7$)-22858 (AR grade, Qualigens Fine Chemicals, Mumbai, India)
4. Sucrose-575001 (AR grade, MP Biomedicals, France)
5. Invertase type VII from baker’s yeast, lyophilized powder ≥ 300 units/mg solid-I4504 (Sigma Aldrich, USA)
6. Glucose oxidase type X from *Aspergillus niger,* lyophilized powder, 100,000-250,000 units/gm solids-G7141 (Sigma Aldrich, USA)
7. Peroxidase from horseradish type VI, essentially salt free, lyophilized powder, 250-300 units/mg solid-P8375 (Sigma Aldrich, USA)
8. Tri-sodium citrate ($Na_3C_6H_5O_7.2H_2O$)-27625 (AR grade, Thermo Fisher Scientific India Pvt. Ltd., Mumbai)
9. Whatman filter paper (No.1) (GE healthcare, UK Ltd.)

3.4.2 Reagent

1. Citrate buffer (0.1 M, pH 5.2):

(A) 0.1 M of citric acid solution was prepared by dissolving 2.101 g citric acid in distilled water and volume made up to 100 ml.

(B) 0.1 M of sodium citrate solution was prepared by dissolving 2.941 g trisoidum citrate in distilled water and volume made up to 100 ml.

18 ml of solution A and 32 ml of solution B were mixed together and volume made up to 100 ml with distilled water.

3.4.3 Method of preparation of sucrose detection strip:

3.4.3.1 Preparation of enzyme solution

Invertase, glucose oxidase and horseradish peroxidase were dissolved in cold (4°C) citrate buffer. The prepared solution was stored at 4°C.

3.4.3.2 Preparation of chromogen solution

The chromogen solution was prepared in distilled water by mixing Chromogen 1 and Chromogen 2 in 4:1 ratio. Individual solutions of Chromogen 1 and Chromogen 2 were stored at 4°C and the chromogen solution was prepared fresh on the day of experiment.

3.4.3.3 Preparation of soaking solution

Mix 1 ml of enzyme solution, 1.5 ml of citrate buffer (pH 5.2) and 2.5 ml of chromogen solution. Mix the content thoroughly using a clean spatula. Soaking solution is to be prepared fresh.

3.4.3.4 Adsorption of reagents on filter paper

The filter paper was pre-extracted in a Soxhlet extractor for 4 h. The extracted filter paper was dried at room temperature in a fume hood, and then cut into 4.0 cm × 2.5 cm^2 strips. Five milliliters of soaking solution was taken in clean dry glass petriplate. The acetone-treated strips were dipped in the soaking solution for at least 4 min. After that strips were taken out and kept for air drying (2 h) in a BOD incubator at 30°C. After complete drying, the test paper was cut into small strips of 1x6 cm^2 size, pasted on an inert paper (7 x 6 cm^2) using a non-reactive adhesive. The inert paper strip along with pasted detection strip was then cut into 0.5×6 cm^2 size and sealed in a plastic bottle and stored at refrigeration temperature.

3.4.4 Proof of principle of working of prepared dry reagent test strip in sucrose added milk

The milk sample was taken in a 100 ml beaker and brought to room temperature. Appropriate amount of sucrose was added to milk sample to reach sucrose concentration at 0.1% level in milk. A drop (10 µl) of control and spiked sample (before and after boiling) was put on test strip and compared for color contrast with that of control milk sample.

3.4.5 Determination of response time

Two samples added with sucrose at 0.1 and 0.12% level were subjected to determine the response time of the strip. Each sample was tested with prepared sucrose detection strip and observed for the optimum color contrast with that of control sample.

3.4.6 Determination of limit of detection of sucrose in milk using prepared test strip

To determine limit of detection of prepared dry reagent strip, the appropriate levels of sucrose was added to milk so as to achieve final sucrose concentration in milk at 0.02, 0.04, 0.06, 0.08, 0.1 and 0.12% levels. Each spiked milk sample was then tested with sucrose test strip and compared for color contrast with that of control sample.

3.5 Qualitative detection of added hydrogen peroxide in milk using dry reagent strip

3.5.1 Chemicals and materials

1. Citric acid ($C_6H_8O_7$)-22585 (AR grade, Qualigens Fine Chemicals, Mumbai, India)
2. Chromogen 1
3. Hydrogen peroxide (30% w/v) (AR grade, Qualigens Fine Chemicals, Mumbai, India)

4. Peroxidase from horseradish type VI, essentially salt free, lyophilized powder, 250-300 units/mg solid-P8375 (Sigma Aldrich, USA)
5. Chromogen 2
6. Tri-sodium citrate ($Na_3C_6H_5O_7.2H_2O$)-27625 (AR grade, Thermo Fisher Scientific India Pvt. Ltd., Mumbai, India)
7. Whatman filter paper (No.1) (GE healthcare, UK Ltd.)

3.5.2 Method of preparation of H_2O_2 detection test strip:

3.5.2.1 Preparation of enzyme solution

Horseradish peroxidase was dissolved in cold (10°C) distilled water. The prepared solution was stored at 4°C.

3.5.2.2 Preparation of chromogen solution

Dissolve 100 mg of Chromogen 2 in 1 ml distilled water.

3.5.2.3 Preparation of soaking solution

Prepare soaking solution by mixing 0.5 ml of enzyme solution, 1.5 ml of citrate buffer (pH 5.2), 1.2 ml Chromogen 1 and 1 ml of chromogen solution. Mix the content thoroughly using a clean spatula. Soaking solution is to be prepared fresh.

3.5.2.4 Adsorption of reagents on filter paper

5 ml of soaking solution was taken in a clean dry petriplate. The paper strips (4.0 cm × 2.5 cm^2) were soaked with the soaking solution for 5 min and dried (2 hours) at 30°C in a BOD incubator. After complete drying, the test paper was cut into small strips of 1x6 cm^2 size, the cut strips were pasted on an inert paper (7 x 6 cm^2) using a non-reactive adhesive. The inert paper strip along with pasted detection strip was then cut into 0.5×6 cm^2 size and sealed in a plastic bottle and stored at room temperature (4-5°C).

3.5.3 Proof of principle of working of prepared dry reagent test strip using H_2O_2 added milk

The milk sample was taken in a 100 ml beaker and brought to room temperature. Appropriate amount of hydrogen peroxide was added to milk sample to reach the final level of 0.01%. Both the control and adulterated milk samples were tested with developed test strip. The test strips were dipped in the above prepared milk samples and the results for the change in color of the strips were compared with that of control milk.

3.5.4 Determination of response time

Sample added with H_2O_2 at 0.01% was subjected to determine the response time of the strip. The sample was tested with strip and observed for the optimum color contrast with that of control sample.

3.5.5 Determination of limit of detection of test strip

To determine the limit of detection of developed dry reagent strip, the appropriate level of hydrogen peroxide was added to milk so as to achieve hydrogen peroxide level in milk at 0.0005, 0.001, 0.01 and 0.1% levels. Each spiked milk sample was then tested with hydrogen peroxide test strip and compared for color contrast with that of control sample.

3.6 Qualitative detection of detergent in milk using dry reagent strip

3.6.1 Chemicals and reagents

1. Methylene blue-39692/01 (Qualigens, Mumbai, India)
2. Labolene (Sigma Aldrich, USA)
3. Cetyl tri ammonium bromide (CTAB)-RM 4867 (Hi Media, Mumbai-India)

3.6.2 Method of preparation of detergent detection strip

3.6.2.1 Preparation of chromogen solution

0.1% solution of CTAB was made. Six different solutions (a, b, c, d, e, f) having different concentrations of labolene and methylene blue was made as shown below.

Methylene blue (A) – 100 mg/ 100 ml
Labolene (B) – 200 mg/ 100 ml

Table 3.1: Solutions having different concentrations of A and B

	a	b	c	d	e	f
A	1	1	1	1	1	1
B	0	0.1	0.2	0.4	0.8	1
H_2O	9	8.9	8.8	8.6	8.2	8

3.6.2.2 Preparation of soaking solution

Soaking solution was made on the day of the experiment by adding 3 ml of solution CTAB.

3.6.2.3 Adsorption of reagents on filter paper

3 ml of soaking solution was taken in a clean dry petri plate. The paper strips (4.0 cm × 2.5 cm^2) were dipped in the soaking solution for 5 min and dried. After complete drying, the test paper was cut into desired shape and pasted on an inert paper with the help of double adhesive tape and finally cut into desired shape and sealed in an air tight plastic bottle.

3.6.2.4 Procedure for detection of detergent in milk using detergent detection strip

To detect detergent in milk by using detergent detection strip, a drop of above made solutions (a, b, c, d, e, f) was applied to the test strip followed by visualization of change in color.

3.7 Qualitative detection of added soymilk by lateral flow assay

3.7.1 Chemicals, materials and equipments

Tetrachloroauric acid trihydrate ($HAuCl_4.3H_2O$) G-4022, trisodium citrate dehydrate C-3434, bovine serum albumin (BSA) A-3059 were purchased from M/s. Sigma Aldrich, USA. HPLC grade concentrated hydrochloric acid (1.93001.2521), nitric acid (1.93006.2521) were purchased from M/s. Merck chemicals, Mumbai. Anti-Soy protein antibody (S2519) produced in rabbit was purchased from M/s. Sigma Aldrich, USA. Affinity purified antibody to rabbit IgG produced in goat was purchased from M/s. KPL (01-15-06). Lateral flow accessories such as: absorbent pad (AP045 27x260 mm), sample pad (GFB-R7, 75x260 mm), conjugate pad (PT-R5 70x260 mm) and nitrocellulose membranes (CNPF-SN12-L2-H50, 8, 10 and 15 µ) were procured from MDI Pvt. Ltd. Ambala. Special design glass ampoules (soda glass) were fabricated from J.K. Scientific, Ambala.

Details of instrumentation facility and other accessories used/utilized in this research work are:

- Nanoparticle Size Analyzer: Zeta Series, Nano sizer (Malvern - Nano ZS90),
- ELISA Reader (Tecan I-Control, Infinite F200Pro),
- Circulating Water bath (Poly Science, USA),
- pH Meter (Thermo orion-420),
- Magnetic Stirrer (Spinot- digital, Tarsons),
- Ultrasonicator (Rho),
- Lateral Flow Strip Printer (Easy printer, Model: LPM -02) (MDI Pvt. Ltd. Ambala),
- Paper strips cutter-Deli 12” (Made in Korea).

3.7.2 Preparation of Gold Nanoparticles (AuNPs)

Preparation of AuNPs

The method described by Liu and Lu (2006) was followed for preparation of gold nanoparticles (AuNPs) in aqueous phase using tri sodium citrate as reducing agent. The method is described below:

Cleaning of Glassware:

Cleaning of glassware is one of the critical steps in the preparation of high-quality nanoparticles. Care was taken to ensure that no contamination was introduced during nanoparticle synthesis. Following protocol was followed for the cleaning of glassware.

1. All the glassware’s (double necked flasks and its stopper, condenser, beakers, pipettes, magnetic bar and storage bottle) were first washed with copious amount of tap water.

2. Then, all these glassware were soaked in liquid detergent followed by thorough washing with tap water.
3. Then, these glassware were filled/soaked with freshly prepared aqua regia (3:1 mixture of concentrated HCl and HNO_3) for at least 30 min.
4. Rinsed with copious amount of distilled water and then Milli-Q water in sequence and then covered with aluminium foil and dried in an oven.

Reagents

A. Stock gold chloride (tetrachloroauric acid trihydrate Mol.Wt.393.83; 200 mM*)* solution: 787.6 mg of $HAuCl_4.3H_2O$ was dissolved in Milli-Q water and volume was made up to 10 ml. The stock solution was stored at room temperature in amber colour bottle.

B. Working gold chloride solution (50 mM): Stock gold chloride solution was diluted four times with Milli-Q water.

C. Trisodium citrate dihydrate (38.8 mM; MW. 294): 114 mg of trisodium citrate dihydrate was dissolved in 10 ml Milli-Q water.

Procedure: Preparation of AuNPs

1. In a 250 ml double neck flask, 99.5 ml of Milli-Q water was added and placed over the magnetic stirrer.
2. The flask was connected to the refluxing condenser through the central neck. Magnetic bar was carefully placed inside the flask via side neck and stoppered.
3. Then 0.5 ml of 200 mM gold chloride solution was added through the side neck and stopper was placed. Heating and stirring was initiated.
4. When gold chloride solution starts boiling (reflux), stopper from the side neck of the flask was removed and quickly 10 ml of 38.8 mM trisodium citrate dihydrate solution was added and stopper placed back.
5. Then boiling and stirring was continued. There was a change of colour from pale yellow to deep red in one minute indicating the formation of colloidal gold nanoparticles.
6. The contents were allowed to reflux for another twenty minutes. Heating was turned off and the content was allowed to cool to room temperature (23-25^{o}C) under constant stirring.

7. AuNPs were collected in a clean glass bottle and stored at refrigeration temperature (4-6°).

3.7.3 Characterization of Gold Nanoparticles

3.7.3.1 Measurement of Absorption Spectra of AuNPs

Absorption spectra of AuNPs were recorded by the method of Li (2011) using UV-Vis Spectrophotometer (Shimadzu UV-2550) in the range of 700 to 400 nm with a band width of 0.5 nm against Milli-Q water as blank.

3.7.3.2 Measurement of Size and Zeta Potential of AuNPs

Size and zeta potential of AuNPs was measured by the method of Saha *et al*., (2007). Dynamic light scattering (DLS) was used for determination of average size and zeta potential of prepared gold nanoparticles using Zeta series nano sizer (Malvern - Nano ZS90).

3.7.4 Conjugation of AuNPs with Antibody

1. 10 ml of AuNPs was taken in 15 ml centrifuge tube.
2. It was centrifuged at 6500Xg for 30 min at 4 °C.
3. The supernatant was removed carefully and the pellet was re-suspended in 4 mL of borate buffer
4. 1 mL of this AuNP was taken and 10 uL of Anti Body (Anti-Soya) was added.
5. It was kept for proper mixing using rotating wheel for 30 minute at room temperature.
6. Fresh 10% BSA in borate buffer was kept ready.
7. The unreacted sites of AuNPs was blocked by adding 100 µl of buffer containing 10% BSA and kept for 15 min using rotator at room temperature.
8. Colloid solution was again centrifuged at 6500Xg for 20 min at 4°C, then supernatant was removed and the pellet was re-suspended in 1 ml buffer with 10% BSA.
9. Fresh 0.1% BSA in borate buffer was kept ready.

10. Colloid solution was again centrifuged at 6500Xg for 20 min at 4°C, then supernatant was removed and the pellet containing antibody-AuNPs conjugate was re-suspended in 250 µl buffer containing 0.1% BSA.
11. The prepared conjugate was transferred to a glass vial (previously cleaned with aqua-regia and dipped into 12 N NaOH overnight and washed with ultra-pure water).
12. This Antibody-AuNPs conjugate was dispensed (4 µl) on conjugate pad and the conjugation was checked by running the strip and using species specific secondary antibody.

Note: Prepare borate buffer carefully, adjust pH 7.5, filter it and store at 4°C. Once the pellet is re-suspended transfer the suspension into fresh tube.

3.7.5 Construction of Lateral Flow Assembly

Construction of lateral flow assembly (competitive format) was done as described below. To facilitate the capillary action and for smooth functioning of the strip, all the pads and membrane were arranged to overlap each other one other so that there was a continuous flow of buffer and sample

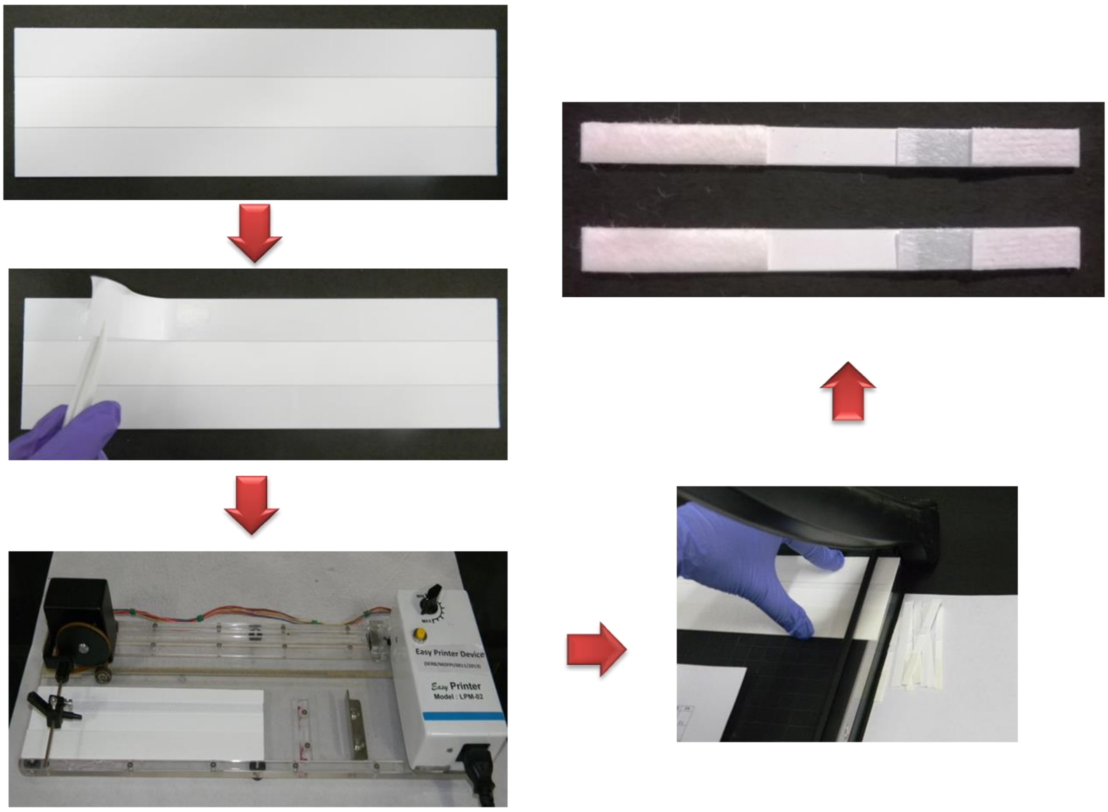


Figure 3.1. Construction of lateral flow strip

Reagents:

A. Anti-Soy Protein antibody produced in rabbit.

B. Goat-antirabbit IgG-antibody- Affinity purified antibodies

C. Antibody conjugated Gold nanoparticles – as optimized in the section 3.6.4

Procedure

1. Preparation of Test matrix (nitrocellulose membrane): With the help of antibody printer assembly (Easy printer); soymilk and goat-anti rabbit IgG-antibody were applied as a thin line and were designated as test line (towards the conjugation pad side) and control line (towards the absorbent pad), respectively on nitrocellulose membrane. The membrane was dried with the help of hair drier.
2. Antibody printed nitrocellulose membrane was taken for the construction of lateral flow assembly. The protective layers from the both ends were peeled off from the backing card to expose the sticky portion.
3. Glass fibre conjugate pad was first pasted on the nitrocellulose membrane at conjugate pad position, so that 2-3 mm of it overlaps on the nitrocellulose membrane.
4. Then sample pad was fixed with 2 mm overlapping on the conjugation pad. Finally the adsorbent pad was fixed to the other side of the nitrocellulose membrane.
5. All these three layers were secured by pressing with the help of peeled off layers.
6. Then it is cut into strips of 5-8 mm each with the help of strip cutter. The strips were stored at room temperature in a box under dry condition. Figure 3.1 shows the steps followed for the construction of lateral flow assembly. Figure 3.2 shows the dimensions of a different components of lateral flow strip.

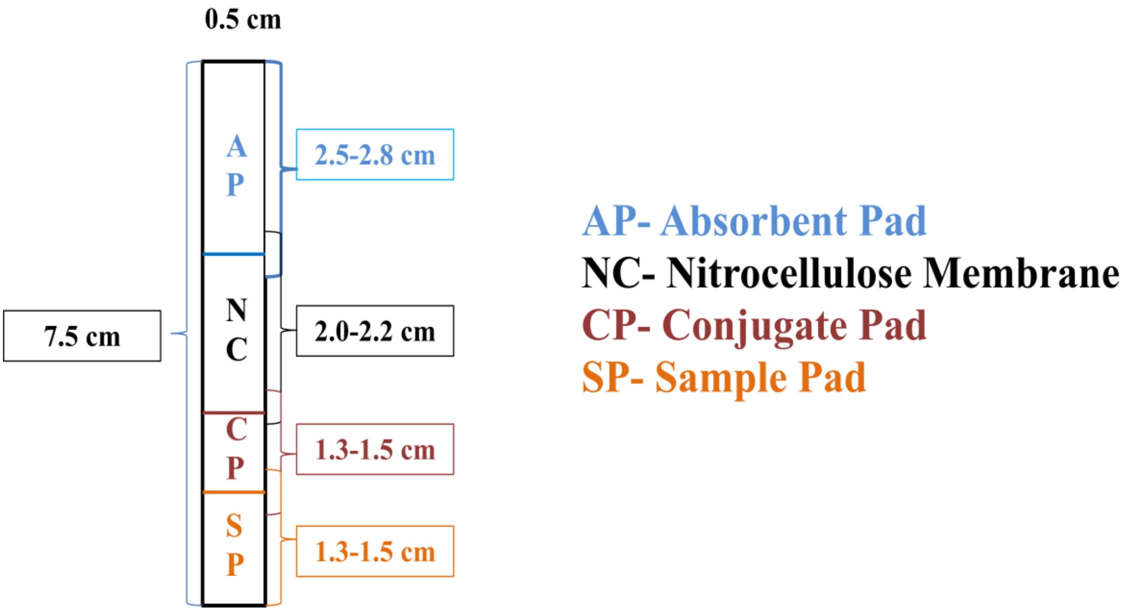


Figure 3.2. Dimensions of a lateral flow strip

3.7.6 Detection of soymilk in Milk Using Lateral Flow Assay

3.7.6.1 Preparation of soymilk

The soymilk was prepared by the method described earlier (Sharma *et al*., 2009). Fifty grams of soybean were soaked in 1000 ml water at room temperature for 10 h. The beans were hand washed to remove their testa. Wet beans were then blended with 500 ml water using kitchen blender for 3 min. The resulting slurry was filtered through two layers of fine muslin cloth and the filtrate was collected in glass beaker. The contents were heated till temperature reached 95°C and then temperature was maintained at 95°C for 10 min. The filtrate was constantly stirred during heating, cooled to room temperature and again filtered through fine muslin cloth. The filtrate, referred as soymilk, was stored at 5°C till further analysis.

Milk Sample Preparation: Fresh milk sample was collected from Cattle Yard, National Dairy Research Institute, Karnal. Milk was centrifuged and skimmed milk was used in the subsequent study. The skim milk was spiked at different levels of soymilk i.e. 0%, 0.25%, 0.5%, 0.75%, 1%, 1.25%, 1.5%, 1.75%, 2%. Same level of spiking of soymilk was done in whole milk and borate buffer. In case of whole milk, the milk sample was spiked at different levels of soymilk i.e. 0%, 0.25%, 0.5%, 0.75%, 1%, 1.25%, 1.5%, 1.75%, 2% followed by the addition of EDTA @ 100 mg/ 100 ml at room temperature, the EDTA supplemented milk was kept at room temperature for 20 min and then was used to apply on the strip.

Test line and control line printed lateral flow assembled strips were taken for the experiment. On the conjugate pad, 7 µl of AuNPs-antibody conjugates was applied. Strips were allowed to dry with the help of hair drier. Then it was tested in skim milk having different concentration of soya milk. Appearance of two lines (i.e. at control as well as test line) indicated that soymilk in sample is below detection limit or absent. Appearance of one line (i.e. at control line) indicated that soymilk was above the detection limit.

3.8 Detection of alkaline phosphatase in milk by dry reagent chemistry

Two techniques were followed for the development of strip for detection of alkaline phosphatase.

1. Using BCIP Red/ NBT solution A

2. Using phenolphthalein diphosphate tetrasodium salt

3.8.1 Using BCIP Red/ NBT Solution A

3.8.1.1 Chemicals

1. BCIP Red/ NBT Solution A-75531 (SRL Chemicals, Maharashtra, India)
2. Tris (hydroxymethyl) aminomethane-154563 (Sigma Aldrich, USA)
3. Hydrochloric Acid-1.93001.2521 (Merck Chemicals, Mumbai-India)

3.8.1.2 Apparatus

1. Test tubes
2. Water bath
3. Pipettes

3.8.1.3 Detection of alkaline phosphatase in liquid phase

Five milliliters of raw milk and pasteurized milk were taken in two different test tubes. To the test tubes 2 ml of BCIP Red/ NBT solution A and 0.5 ml of

Tris-HCl buffer (pH-8.0) was added. The tubes were left undisturbed for the visualization of any color development in the test tubes.

3.8.1.4 Method of preparation of alkaline phosphatase detection strip

3.8.1.4.1 Adsorption of reagents on the filter paper

Five milliliters of BCIP Red/ NBT Solution A and 0.5 ml of Tris-HCl buffer (pH- 8.0) was poured into a petri plate. A piece of filter paper (6×4 cm^2 size) was dipped in it for at least 5 min and then kept for drying. After complete drying the strips were cut into desired shape and pasted on an inert paper with the help of double adhesive tape and finally cut into desired shape and sealed in an air tight plastic bottle.

3.8.1.4.2 Procedure for detection of alkaline phosphatase in milk

A drop of raw and pasteurized milk (8µl) was added to the strips and was observed for the change in color.

3.8.2 Using phenolphthalein diphosphate tetrasodium salt

3.8.2.1 Chemicals

1. Phenolphthalein diphosphate tetrasodium salt-RM1141 (Hi Media, Mumbai-India)
2. Tris (hydroxymethyl) aminomethane-154563 (Sigma Aldrich, USA)
3. Hydrochloric Acid-1.93001.2521 (Merck Chemicals, Mumbai-India)

3.8.2.2 Apparatus

1. Test tubes
2. Water bath
3. Pipettes

3.8.2.3 Detection of alkaline phosphatase in liquid phase

Five milliliters of raw milk and pasteurized milk were taken in two different test tubes. To the test tubes 3 ml of phenolphthalein diphosphate tetrasodium salt and 0.5 ml of Tris-HCl buffer (pH-8.0) was added. The tubes were left undisturbed for the visualization of any color development in the test tubes.

3.8.2.4 Method of preparation of alkaline phosphatase detection strip

3.8.2.4.1 Adsorption of reagents on the filter paper

Five millilitres of phenolphthalein diphosphate tetrasodium salt solution and 0.5 ml of Tris-HCl buffer (pH- 8.0) was poured into a petri plate. A piece of filter paper (6×4 cm^2 size) was dipped in it for at least 5 min and then kept for drying. After complete drying the strips were cut cut into desired shape and pasted on an inert paper with the help of double adhesive tape and finally cut into desired shape and sealed in an air tight plastic bottle.

3.8.2.4.2 Procedure for detection of alkaline phosphatase in milk

A drop of raw and pasteurized milk (8µl) was added to the strips and was observed for the change in color.

3.9 Detection of lactoperoxidase in milk by dry reagent chemistry

Two techniques were followed for the development of strip for detection of alkaline phosphatase.

1. Using TMB liquid substrate for membranes.
2. Using 4-AAP and phenol

3.9.1 Using TMB liquid substrate for membranes.

3.9.1.1 Chemicals

1. TMB liquid substrate for membranes-T0565. (Sigma Aldrich, USA)
2. Citric acid ($C_6H_8O_7$)-22858 (AR grade, Qualigens Fine Chemicals, Mumbai, India)
3. Tri-sodium citrate ($Na_3C_6H_5O_7.2H_2O$)-27625 (AR grade, Thermo Fisher Scientific India Pvt. Ltd., Mumbai)

3.9.1.2 Reagents

1. Citrate buffer (0.1 M, pH 5.2):

(A) 0.1 M of citric acid solution was prepared by dissolving 2.101 g citric acid in distilled water and volume made up to 100 ml.

(B) 0.1 M of sodium citrate solution was prepared by dissolving 2.941 g trisodium citrate in distilled water and volume made up to 100 ml.

18 ml of solution A and 32 ml of solution B were mixed together and volume made up to 100 ml with distilled water.

3.9.1.3 Apparatus

1. Test tubes
2. Water bath
3. Pipettes

3.9.1.4 Detection of lactoperoxidase in liquid phase

Five milliliters of raw milk and milk heated at 80°C were taken in two different test tubes. To the test tubes 1 ml of TMB and 0.5 ml of citrate buffer (pH-5.2) was added. The tubes were left undisturbed for the visualization of any color development in the test tubes.

3.9.1.5 Method of preparation of lactoperoxidase detection strip

3.9.1.5.1 Adsorption of reagents on the filter paper

5 ml of TMB and 2 ml of citrate buffer (pH-5.2) was poured into a petri plate. A piece of filter paper (6×4 cm^2 size) was dipped in it for at least 5 min and then kept for drying. After complete drying the strips were cut into desired shape and pasted on an inert paper with the help of double adhesive tape and finally cut into desired shape and sealed in an air tight plastic bottle.

3.9.1.5.2 Procedure for detection of lactoperoxidase in milk

A drop of raw and milk heated to 80°C (8µl) was added to the strips and was observed for the change in color.

3.9.2 Using 4-AAP and Phenol.

3.9.2.1 Chemicals

1. 4-amino antipyrine-A4382 (Sigma Aldrich, USA)
2. Tris (hydroxymethyl) aminomethane-154563 (Sigma Aldrich, USA)
3. Hydrochloric Acid-1.93001.2521 (Merck Chemicals, Mumbai-India)

4. Phenol-P1037 (Sigma Aldrich, USA)
5. Hydrogen peroxide (30% w/v) (AR grade, Qualigens Fine Chemicals, Mumbai, India)

3.9.2.2 Reagents

1. Preparation of aminoantipyrine and phenol mix: Aminoantipyrine (0.0203 g) and phenol (0.056 g) were dissolved in 100 ml of 0.1 M Tris–HCl buffer (pH 7.7). The contents were mixed. The prepared aminoantipyrine and phenol mix solution was stored at 4 °C.

3.9.2.3 Apparatus

1. Test tubes
2. Water bath
3. Pipettes

3.9.2.4 Detection of lactoperoxidase in liquid phase

Two hundred micro liters of milk sample was added to 2 ml of aminoantipyrine and phenol mix solution. The contents were vortexed briefly followed by the addition of 20 µl of hydrogen peroxide solution (30%). The contents were mixed, incubated at 37 °C for 10 min and color of the solution was noted.

3.9.2.5 Method of preparation of lactoperoxidase detection strip

3.9.2.5.1 Adsorption of reagents on the filter paper

Three milliliters of amino antipyrine-phenol mixture and 2 ml of Tris-HCl buffer (pH-7.7) was poured into a petri plate. A piece of filter paper (6×4 cm^2 size) was dipped in it for at least 5 min and then kept for drying. After complete drying the strips were cut into desired shape and pasted on an inert paper with the help of double adhesive tape and finally cut into desired shape and sealed in an air tight plastic bottle.

3.9.2.5.2 Procedure for detection of lactoperoxidase in milk

A drop of raw and milk heated to 80°C (8µl) was added to the strips and was observed for the change in color.

3.10 Validation of the test strips in milk products

3.10.1 Validation of glucose, sucrose, maltodextrin detecting paper strips in *Dahi*

3.10.1.1 Preparation of *dahi* spiked with glucose, sucrose and maltodextrin

Raw Milk was procured from NDRI cattle yard. Milk was divided into three different batches (A, B, C) of 100 ml and it was spiked at two different levels of the above mentioned adulterants i.e. glucose, sucrose, Maltodextrin @ 0.5 and 1% levels. Glucose, Sucrose, Maltodextrin was added to milk samples named as A, B, C respectively. *Dahi* was prepared by procuring the *dahi* culture (167) from NCDC, NDRI Karnal. *Dahi* was prepared by the method prescribed by De (1991). The prepared *dahi* was then checked for the presence or the absence of the spiked adulterants.

3.10.2 Validation of glucose, sucrose, maltodextrin detecting paper strips in *khoa*

3.10.2.1 Preparation of *khoa* spiked with glucose, sucrose and maltodextrin

Raw Milk was procured from NDRI cattle yard. Milk was divided into three different batches (A, B, C) of 100 ml and it was spiked at two different levels of the above mentioned adulterants i.e. glucose, sucrose, maltodextrin @ 0.5 and 1% levels. Glucose, Sucrose, Maltodextrin was added to milk samples named as A, B, C respectively. *Khoa* was prepared by the method prescribed by De (1991). The prepared *khoa* was then checked for the presence or the absence of the spiked adulterants.

CHAPTER – 4

Results and Discussion

4. RESULTS AND DISCUSSION

The results obtained from different sets of experiment carried out according to the objective mentioned in Chapter 1 and following methods as described in Chapter 3 are presented in this Chapter.

4.1 Neutralizer detection strip:

4.1.1 Working of the neutralizer detection strip

The neutralizer detection strip was developed based on the principle that addition of neutralizers to milk would change the natural pH of the milk. Therefore, a suitable pH sensitive chromogen was adsorbed on a filter paper for the development of neutralizer detection strip. The prepared strip was yellow in color. The working of the strip was checked by observing the change in color of the strip when dipped in control as well NaOH neutralized milk samples (at 0.1% level). Figure 4.1 represents the difference in color of the strip when used for control and NaOH containing milk samples.

In the positive milk sample, the color of the strip changed to green while in case of control milk sample, the strip color remained original i.e. yellow color.

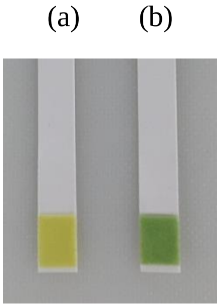


Figure 4.1. Working of neutralizer detection strip. (a) in control milk sample, strip colour does not change (b) in neutralizer (NaOH at 0.1% level) adulterated milk sample strip colour changes to green.

The binding of the reagent on the strip was satisfactory as leaching of reagent into milk sample was not observed. Moreover, the flow of milk on the strip during diffusion did not carry away the reagents on the filter paper thus indicating the stability of reagent on the strip.

4.1.2 Determination of response time of neutralizer detection strip

The test strip was able to respond immediately when came in contact with neutralized milk samples. As observed, change in color was within a fraction of second, the response time was considered as less than one second and thus recommended to observe the color immediately. The color on the strip was stable for about 8-10 min.

4.1.3 Determination of LOD of neutralizer detection strip

The milk sample was neutralized individually with NaOH, Na_2CO_3 and $NaHCO_3$ at 0.01, 0.03, 0.05 and 0.07% levels for NaOH, 0.01, 0.03, 0.05, 0.07 and 0.09% levels for Na_2CO_3, 0.01, 0.03, 0.05, 0.07, 0.09 and 0.1% levels for $NaHCO_3$. At each level, the pH of milk sample was measured to check the minimum level of pH which can be detected by the test strip. The natural pH of milk was found 6.62 and the measured pH of the milk containing different levels of neutralizers is shown in Table 4.1. The results are also represented graphically in Figure 4.2

Table 4.1. **pH of milk containing individual neutralizers (NaOH, Na_2CO_3 and $NaHCO_3$) at different levels.**

Neutralizer level (%) in milk	**pH of milk**		
	NaOH	**Na_2CO_3**	**$NaHCO_3$**
0	6.62	6.62	6.62
0.01	6.75	6.71	6.65
0.03	**7.09**	6.92	6.77
0.05	7.42	**7.1**	6.91
0.07	7.74	7.39	7.03
0.09	8.06	7.57	7.18
0.1	8.52	7.71	**7.29**

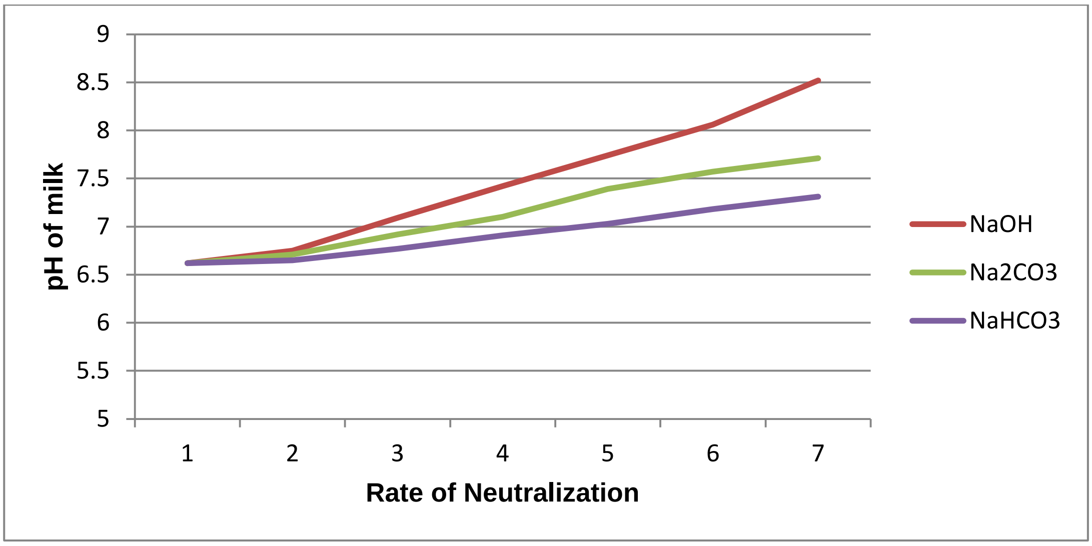


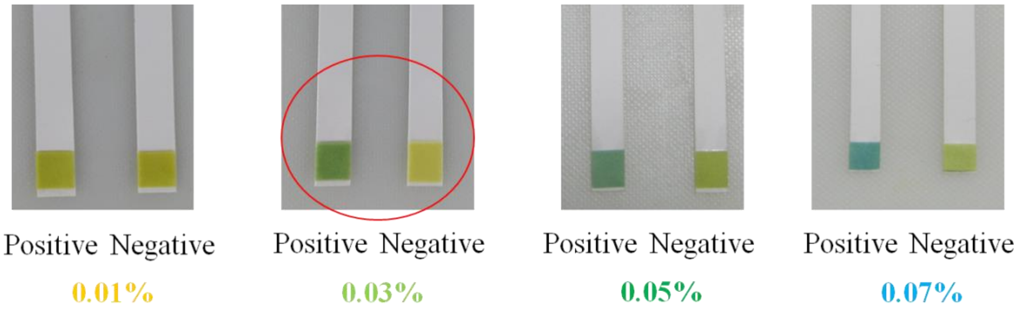
Figure 4.2. Effect of neutralization on pH of milk.

The results indicated that the rate of increase in pH is due to addition of NaOH in milk and was higher than those of Na_2CO_3 and $NaHCO_3$.

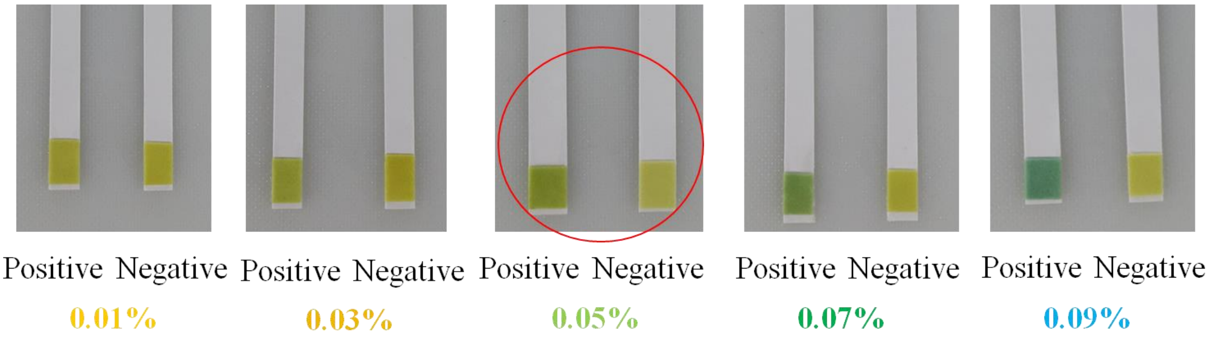
Figure 4.3 shows the change in color of neutralizer detection strip when checked with control milk and various neutralizer containing milk samples at various levels. The results indicated that the perceptible change in color of strip occurred in milk sample containing NaOH at 0.03% level and thus this was considered as LOD of the developed strip. LOD for Na_2CO_3 and $NaHCO_3$ in milk using the developed strip was found 0.05 and 0.1%, respectively. The pH of the milk at LOD level was 7.09, 7.1 and 7.29 for NaOH, Na_2CO_3 and $NaHCO_3$, respectively. In the Table 4.1, the red marked fonts indicate a minimum level of pH at which the test strip was able to show a detectable color change.

It was concluded that if the milk pH has reached ≥ 7.09 on addition of neutralizers, the developed strip can detect such change in pH. Therefore the developed neutralizer detection strip can find application in detection of neutralizers in milk even at low level. It may be further mentioned, since the strip based method is based on the pH of milk, the sensitivity of the method will differ in case of detection of neutralizers in acidic milk. However, it is worth mentioned here that method is more sensitive than the existing rosalic acid test. In our laboratory, the sensitivity of the rosalic acid test was found to be 0.1, 0.1 and 0.2% for NaOH, Na_2CO_3 and $NaHCO_3$ while that of developed strip was better.

a. Working of the neutralizer detection strip with milk samples neutralized with NaOH at various levels



b. Working of the neutralizer detection strip with milk samples neutralized with Na_2CO_3 at various levels



c. Working of the neutralizer detection strip with milk samples neutralized with $NaHCO_3$ at various levels

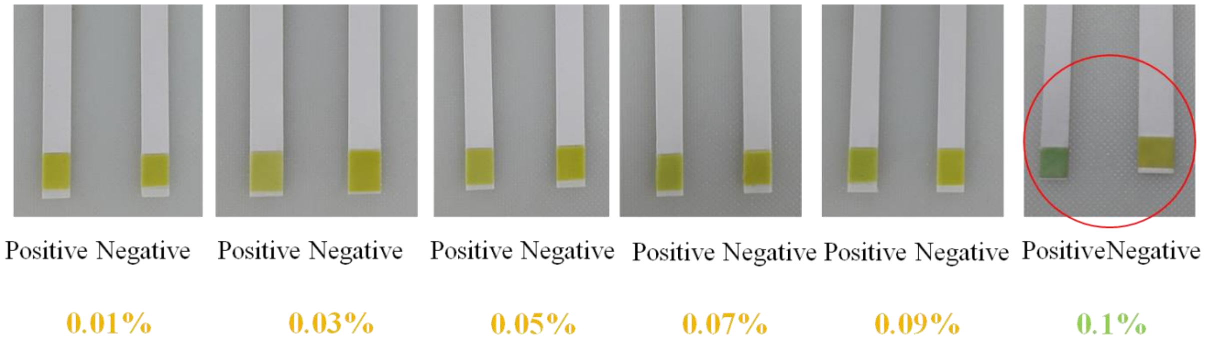


Figure 4.3. Study of limit of detection of neutralizer detection strip with various neutralizers in milk.

4.1.4 Storage study of neutralizer detection strip

The prepared strip was stored at room temperature (15-30°C) in a screw tight plastic container. The strip was analyzed at monthly intervals. Shelf life of the strip was observed to be 8 months at room temperature. There was no change in the color of the strip during storage. The differentiation between negative control and positive control (at limit of detection) was not affected up to 8 months.

4.2 Urea detection strip

4.2.1 Working of urea detection strip

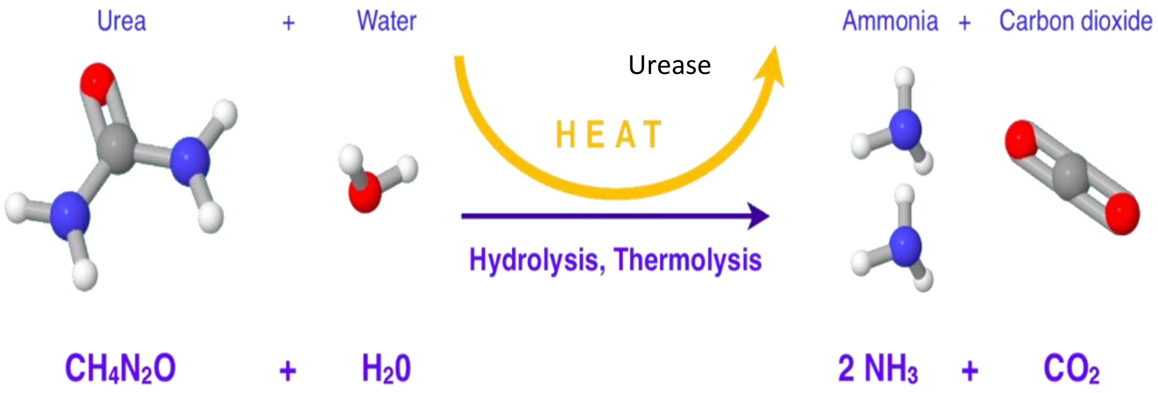


Figure 4.4. Hydrolysis of urea by urease

The urea detection strip was developed by immobilizing urease along with chromogen. On contact with milk, urease degrades the urea in milk and resulting products increase the pH of milk. The increase in milk pH is sensed by chromogen which changes its color. In the presence of extraneously added urea in milk, the color of chromogen present on the test strip turned to red. The original color of the strip is yellow which turns to red when tested with positive milk samples. According to the mechanism of working of the test strip, the intensity of red color produced in the strip would be proportional to the amount of urea present in milk sample. Figure 4.4 depicts the hydrolysis of urea by urease.

The working of test strip was analyzed in milk sample added with 0.1% urea. The result is presented in Figure 4.5 and according to it, in control milk sample test strip remained yellow in color while in urea added milk sample, it changed to red color.

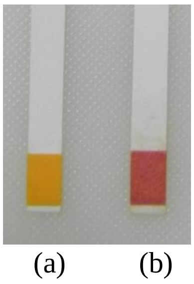


Figure 4.5. Working of urea detection strip. (a) in control milk sample strip colour does not change (b) in urea (at 0.1% level) added milk sample strip colour changes to deep red.

Though normal milk also contains natural urea, the test strip tested with control sample remained yellow. This is because; the amount of urea naturally present in milk is of small quantity (18 - 40 mg/100 ml) compared to that of adulterated milk samples (Sharma *et al*., 2008, Ohlsson *et al.,* 2010). The working of the strip has been optimized in such a way that strip color would not change in presence of normal level of urea in milk.

4.2.2 Determination of response time of urea detection strip

To determine the response time, two milk samples were prepared by adding urea at 0.1 and 0.12% level. These samples along with control milk samples were analyzed with the test strip and increase in the intensity of red color on the strip was observed. The results are presented in Figure 4.6. Since working of the strip involved hydrolysis of urea followed by sensing of change in pH by chromogens, the development of color takes some time and color development is slow. It took a few min to develop a uniform color on the strip. In both types of prepared milk samples (containing externally added urea at 0.1 and 0.2% level), the strip was able to recognize the adulterated milk samples within 3 min. So the response time of the strip was considered as 2 min and therefore the result should be interpreted after 3 min of dipping the test strip in milk samples.

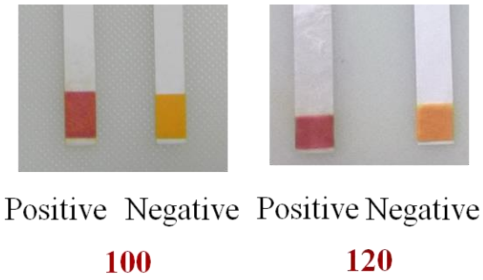


Figure 4.6. Study of response time of urea detection strip in milk samples containing urea at two different levels (100 mg/ 100 ml of milk and 120 mg/ 100 ml of milk).

4.2.3 Determination of LOD of urea detection strip

The LOD of urea detection strip was estimated based on total urea content of the milk i.e. natural urea plus added urea in milk. Urea was added to cow milk sample to make up different level of total urea content in milk varying from 40 to 120 mg per 100 ml. Each sample was then tested with the test strip and compared with that of control milk sample for optimum color contrast. The results obtained are presented in the Figure 4.7. Up to 60 mg/100 ml of total urea in milk, no difference in the color of the strip was observed. At 80 mg per 100 ml level of urea in milk, a distinct color difference was observed which was easily detectable. Therefore, LOD of urea detection strip was considered as 80 mg of total urea per 100 ml of milk sample. As per Food Safety Standards Regulations, 2011 (FSSR, 2011), urea content in milk should not be more than 70 mg/100 ml. The developed strip does not change color at this level. Therefore, the developed strip can find application in detection of extraneously added urea in milk.

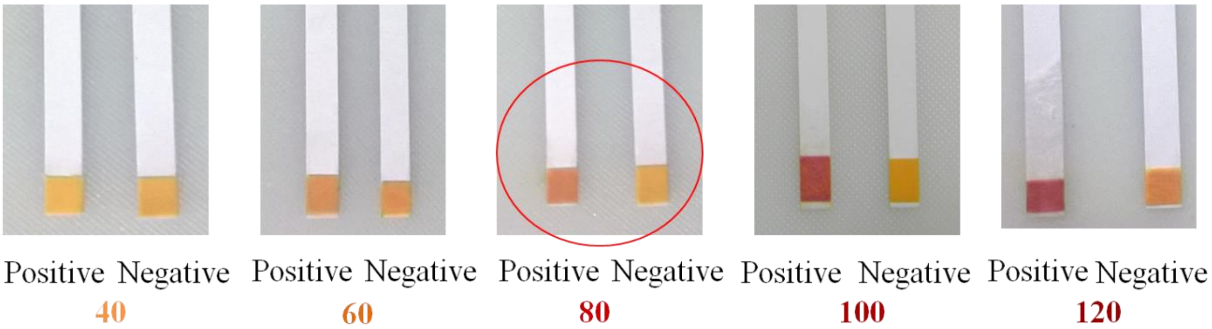


Figure 4.7. Study of limit of detection of urea detection strip. The numerical values indicate the total urea content (natural + added) in milk in mg/100 ml.

4.2.4 Storage study of urea detection strip

The urea strips after preparation was stored in an air tight plastic container at 4°C. The shelf-life of the strip was satisfactory for five months at 4°C. No change in the color of the strip during storage was observed. Differentiation between negative control and positive control (at limit of detection) was not affected up to 4 months.

4.3 Sucrose detection strip:

4.3.1 Working of sucrose detection strip

The sucrose detection strips were developed based on the principle of combined effect of invertase, glucose oxidase and peroxidase reaction. The reaction mechanism of sucrose detection strip involved four steps (i) sucrose in the presence of invertase is hydrolyzed into glucose and fructose (ii) glucose in presence of glucose oxidase reacts with oxygen and water to produce hydrogen peroxide (iii) the formed hydrogen peroxide reacts with TBHBA (2,4,6- tribromo 3-hydroxy benzoic acid) to produce 2,4-dibromo-3,6-dioxocyclohexa-1,4-

dienecarboxylic acid (iv) which in turn reacts with 4-APP (4-amino antipyrine) to yield a red colour compound. Figure 4.8 shows the reaction mechanism for the detection of sucrose. The test strip developed had two key-components, namely chromogen and enzymes. Invertase, glucose oxidase and peroxidase are the commonly used enzymes to catalyze the reaction of sucrose with the chromogen agent. These enzymes work in a narrow pH range of 4–5. Identification of an appropriate chromogen agent is one of the critical steps in the development of paper test strips for measuring sucrose concentrations. There are different chromogen agents available, but most of them are generally not suitable for paper strips. For example, DCP (2, 4-dichlorophenol) and TMB (3, 3’, 5, 5’-tetramethyl benzidine) have too low solubility in water (e.g. DCP: 0.44 g/l at pH 5; TMB: 0.075 g/l at pH 5) in the pH range interested in this study. In contrast, TBHBA has good water solubility i.e. 90 g/l at pH 5. More importantly, TBHBA can be attached/fixed firmly onto the cellulose fibers of the paper substrate via charge interaction; due to the fact that cellulose fibers bear negative charges and TBHBA/4-APP do have positive charges (Ruitao *et al.*, 2012).

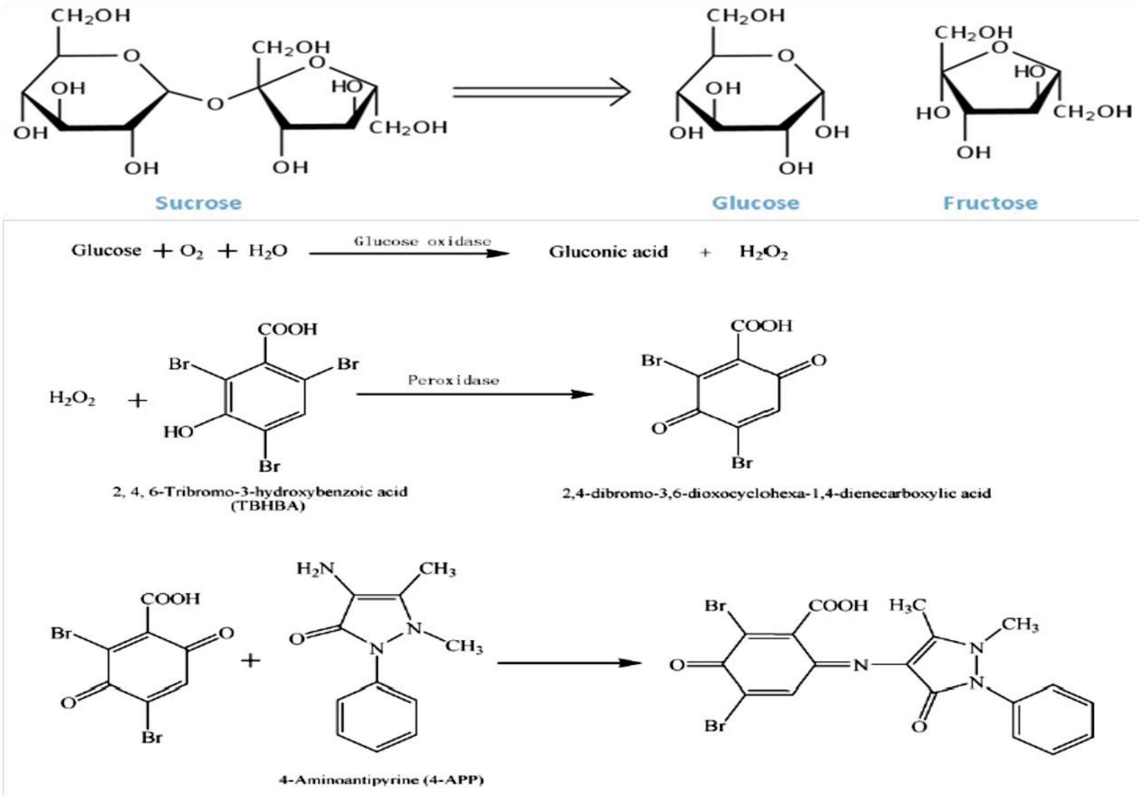


Figure 4.8. Mechanism for the detection of sucrose in milk

In the presence of sucrose in milk, the color of chromogen present on the test strip turned to red. The original color of the strip is white which turn to red when tested with positive milk samples. According to the mechanism of working of the test strip, the intensity of red color produced in the strip would be proportional to the amount of sucrose present in milk sample.

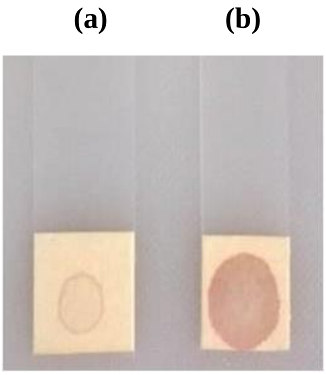


Figure 4.9. Working of sucrose detection strip. (a) In control milk sample strip color does not change (b) in sucrose (at 0.1% level) adulterated milk sample strip color changes to red.

In case of sucrose detection strip, it is advisable that a drop (10 µl) of milk sample should be applied and strip should not be dipped in milk sample as in the latter case, the color uniformity is not there and thus visual interpretation is affected.

4.3.2 Determination of response time of sucrose detection strip

To determine the response time of sucrose detection strip, milk sample was adulterated with sucrose at 0.1 and 0.12% level. The sample was tested with test strip and observed for the time at which detectable color change was obtained on test strip. The results obtained are presented in Figure 4.10. The test strip is able to give perceptible color change within three min therefore the response time was considered as 3 min.

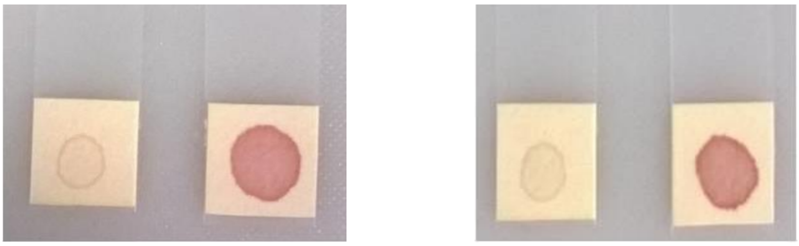


Figure 4.10. Study of response time of sucrose detection strip. The study was done in two milk samples containing 0.1% and 0.12% added sucrose. In each pair, the left hand side of the strip indicated control milk sample while right hand side strip indicated milk sample added with sucrose.

Therefore, the positive or negative results should be interpreted after 3 min of putting the drop of milk sample on test strip.

4.3.3 Determination of LOD of sucrose detection strip

The milk sample was added with sucrose from 0.02 to 0.12% level. Each sample was then tested with the strip and compared with that of control sample as presented in the Figure 4.11. Upto 0.08% of added sucrose the produced H_2O_2 is not sufficient to develop an intense color. But, at the level of 0.1% or more it is enough to develop a detectable color contrast with respect to control. Therefore, the limit of detection was accepted as 0.1% of added sucrose.

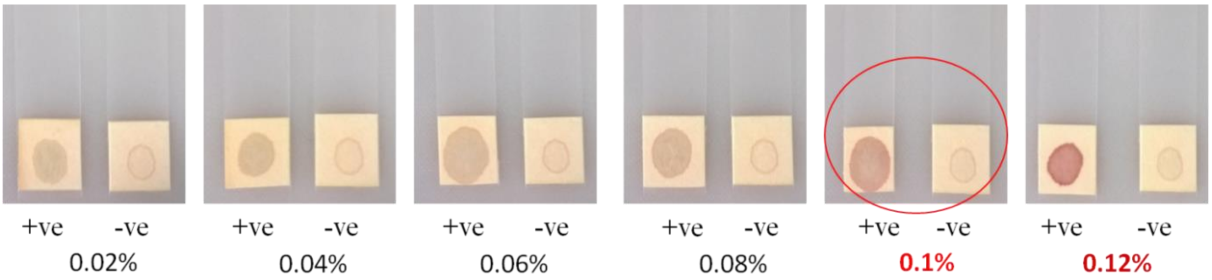


Figure 4.11. Study of limit of detection of sucrose detection strip

4.3.2 Storage study of sucrose detection strip

The prepared strip was packed in air tight plastic containers and stored at refrigeration temperature (4°C) to analyze the storage profile. Shelf life of the strip was observed to be 1 month at refrigeration temperature. The color of the strip changed from white to intense yellow after one month of storage. Differentiation between negative control and positive control (at limit of detection) was not affected up to 1 month.

4.4 Hydrogen peroxide detection strip:

4.4.1. Working of the hydrogen peroxide detection strip

The principle of working of this strip was based on the liberation of iodine from potassium iodide in the presence of hydrogen peroxide catalyzed by enzyme peroxidase. The prepared test strip was validated with the raw milk sample added with H_2O_2 (30% solution) at 0.01% level and results are presented in Figure 4.12. The change in color is noticed in case of positive milk sample and strip color changed to brown. In case of control milk samples, the strip color remained colorless.

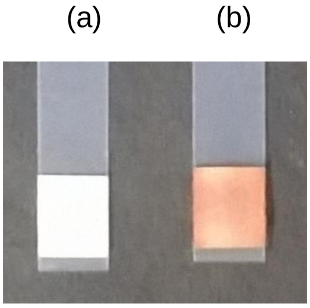


Figure 4.12. Working of hydrogen peroxide detection strip. (a) in control milk sample strip colour does not change (b) in hydrogen peroxide (at 0.01% level) adulterated milk

4.4.2 Determination of response time of hydrogen peroxide detection strip

The response time of the strip was determined by addition of H_2O_2 (30% solution) at 0.01% level. The results of the study are presented in Figure 4.13. The test strip was able to respond immediately when came in contact with the adulterated milk samples. As observed, change in color was within a fraction of second, the response time was considered as less than one second and thus recommended to observe the color immediately.

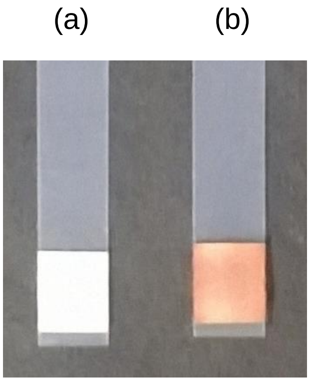


Figure 4.13. Study of response time of hydrogen peroxide detection strip in milk. (a) pure milk sample (b) Milk adulterated with 0.01% of hydrogen peroxide.

4.4.3 Determination of LOD of hydrogen peroxide detection strip

For determination of LOD of the test strip, four milk samples were prepared, spiked with H_2O_2 at different level i.e. 0.0005, 0.001, 0.01 and 0.1%. Each milk sample was analyzed with test strip and observed immediately for the optimum detectable color change. The minimum concentration of hydrogen peroxide which could be detected by the strip came to be 0.001% of 30% H_2O_2.

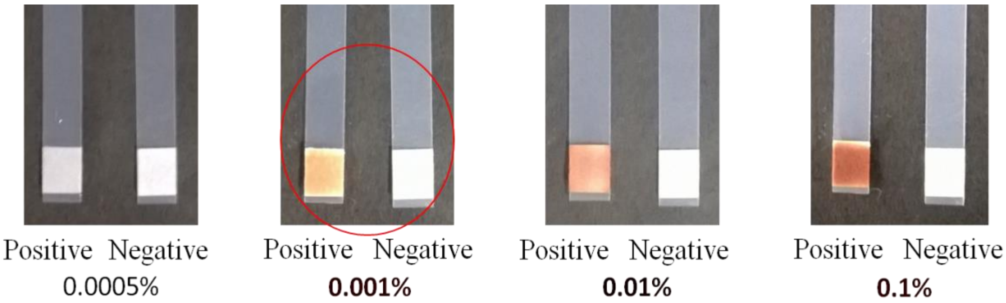


Figure 4.14. Study of limit of detection of hydrogen peroxide detection strip.

4.4.4 Storage study of hydrogen peroxide detection strip

The prepared strip was stored at temperatures refrigerated temperature (4°C) in air tight plastic containers. Shelf life of the strip was observed to be 5 months at refrigeration temperature. There was no change in the color of the strip during storage was observed. The differentiation between negative control and positive control (at limit of detection) was not affected up to 5 months.

4.5 Detergent detection strip:

4.5.1 Working of detergent detection strip

The detection of detergent in milk is based on the principle that anionic detergent if present in the milk sample will compete with methylene blue added to milk for the cationic detergent immobilized on the paper strip. It was hypothesized that the strip when applied to the milk sample its color intensity (due to methylene blue) will decrease as the concentration of the anionic detergent increases in the milk sample. To validate the proper working of the proposed hypothesis solution of various concentrations of methylene blue, anionic detergent was made in water as discussed in Chapter 3. No satisfactory results were obtained as the distinction between pure and detergent spiked milk could not be made. After a number of trials it was decided to stop the further work on the detergent detection strip. Figure 4.15 shows the color developed on the strip when it was applied to the above mentioned solutions.

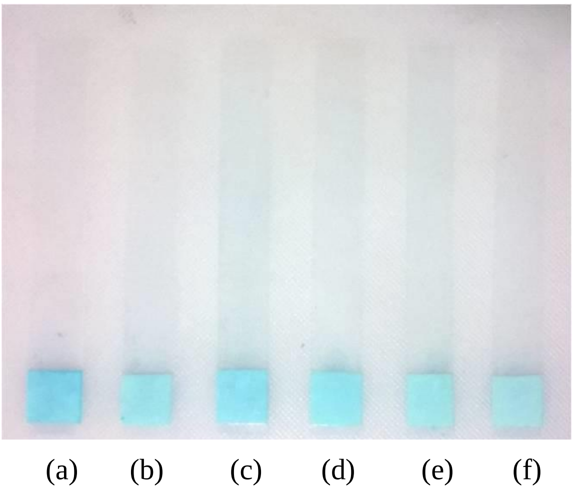


Figure 4.15. Working of detergent detection test strip. The results were inconclusive and thus further work was abandoned.

4.6 Detection of soymilk by lateral flow assay

Lateral flow assay (LFA) is an immunological technique wherein interaction between antibodies and analyte are visualized with the help of gold nanoparticles. Development of good quality antibodies is a crucial step for the success of gold nanoparticles. In the present study, commercially available antibodies against soy protein were used for the development of LFA for detection of soymilk in milk. The competitive format of LFA was used in the present study. The concept of competitive format of lateral flow assay strip is depicted in (Figure 4.16)

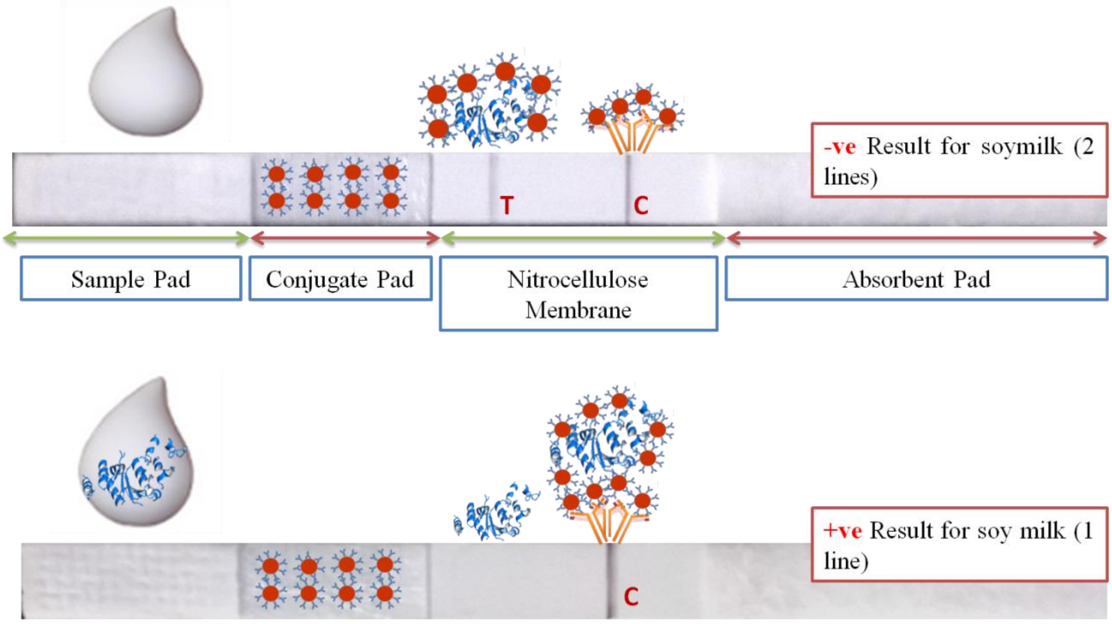


Figure 4.16. Competitive format for Lateral flow assay

4.6.1 Preparation of gold nanoparticles (AuNPs) and their characterization

Nanoparticles are typically in the size range of 1-100 nm. AuNPs required for LFA were prepared in the lab using citrate reduction method as suggested by Liu and Lu (2006). (Figure 4.17) shows the change in color from pale yellow - dark violet - cherry red after addition of reducing agent citrate. The reducing agent should be added as rapidly as possible in order to accomplish a nearly mono dispersed solution. The shape, size, and stability of the colloidal gold particles are crucial factors for the success of the lateral flow strip. Following reaction takes place during the formation of AuNPs (Li *et al*., 2011).

$$6\mathrm{AuCl_4^-} + \mathrm{C_6H_8O_7} + 5\mathrm{H_2O} \rightarrow 6\mathrm{CO_2} + 24\mathrm{Cl^-} + 6\mathrm{Au^0} + 18\mathrm{H^+}$$

The AuNPs prepared in the present study by citrate reduction method were characterized by different techniques [spectroscopy, DLS (for their size distribution and for zeta potential)].

4.6.2 Optical properties of AuNPs

AuNPs exhibit strong absorption of electromagnetic waves in the visible range, due to plasmon resonance. Plasmon resonance is caused due to collective oscillations of the conduction electrons of AuNPs upon irradiation with visible light (Zhao *et al*., 2008). In present study characteristic plasmon resonance peak has been observed at 520 nm with absorbance of 2.1247 (Figure 4.18). In general, it has been reported that absorption maximum shifts to longer wavelengths with increasing particle size (Cai *et al*., 2008; Chun, 2009). Further, the relative uniformity of the particles or the range of particles can be gauged by the width of the absorption spectra: the sharper the band, the more uniform the particles (Chun, 2009).

4.6.3 Measurement of size and zeta potential of prepared AuNPs using dynamic light scattering (DLS)

DLS is a technique that is widely used to characterize size of various particles including proteins, polymers, micelles, carbohydrates, and nanoparticles. It measures the scattering of light by particles in solution. As the intensity of the light scattered is proportional to the six power of the particle size, larger particles will give a greater signal than smaller particles (Thobani, 2010). It is a fast technique to determine the presence or absence of aggregates in AuNPs with low volume requirement. Each sample was measured in triplicate and the mean value has been reported. The average size of prepared AuNPs was 28.59 nm as determined by DLS (Figure 4.19). The synthesized AuNPs have a zeta potential of -38.3 mV (Figure 4.20) confirming their stability as particles with zeta potentials more than +30 mV and more negative than -30 mV are normally considered stable (Nara *et al*., 2010).

Figure 4.17. Color changes during preparation of AuNPs

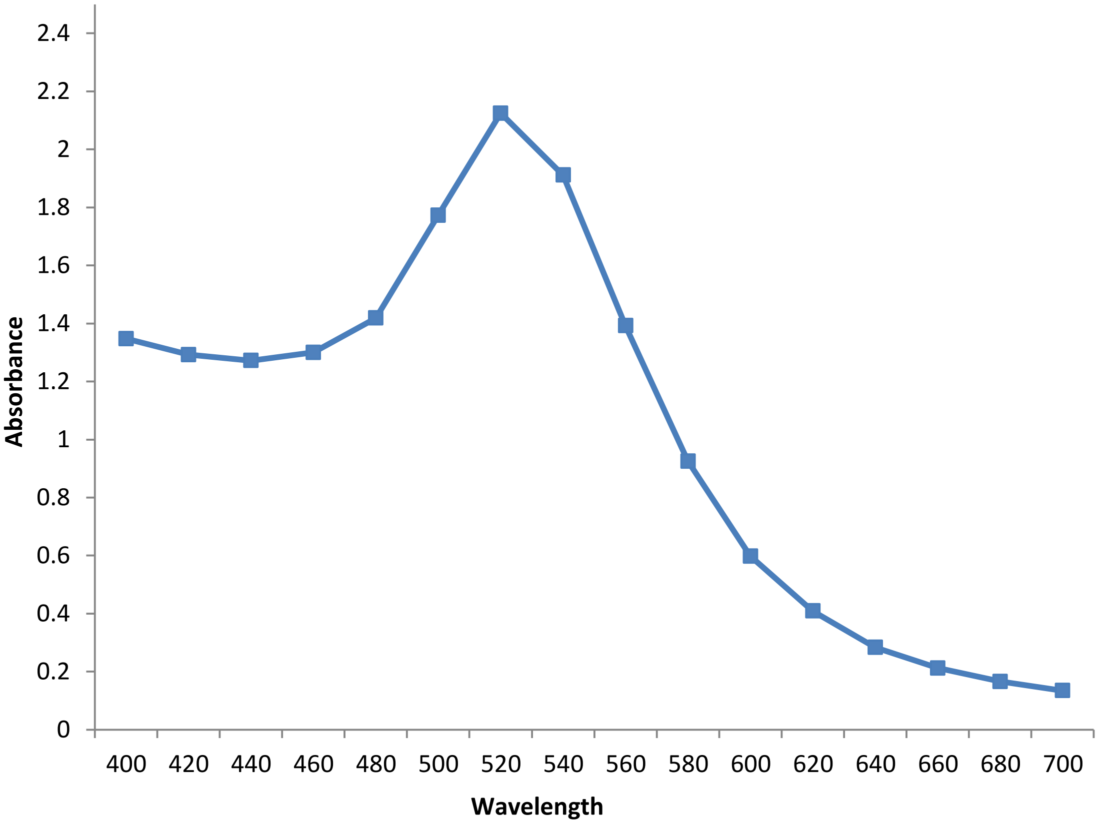
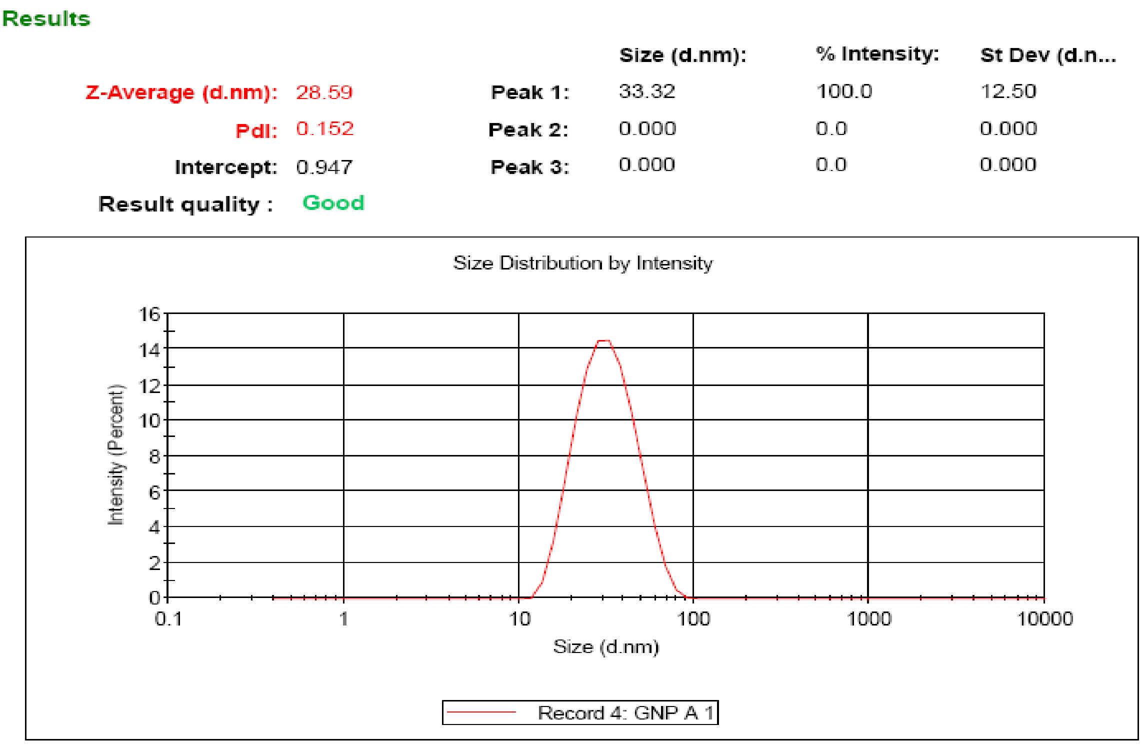


Figure 4.18. Absorption spectra of AuNPs prepared in the laboratory by citrate reduction method



Results

Z-Average (d.nm): 28.59

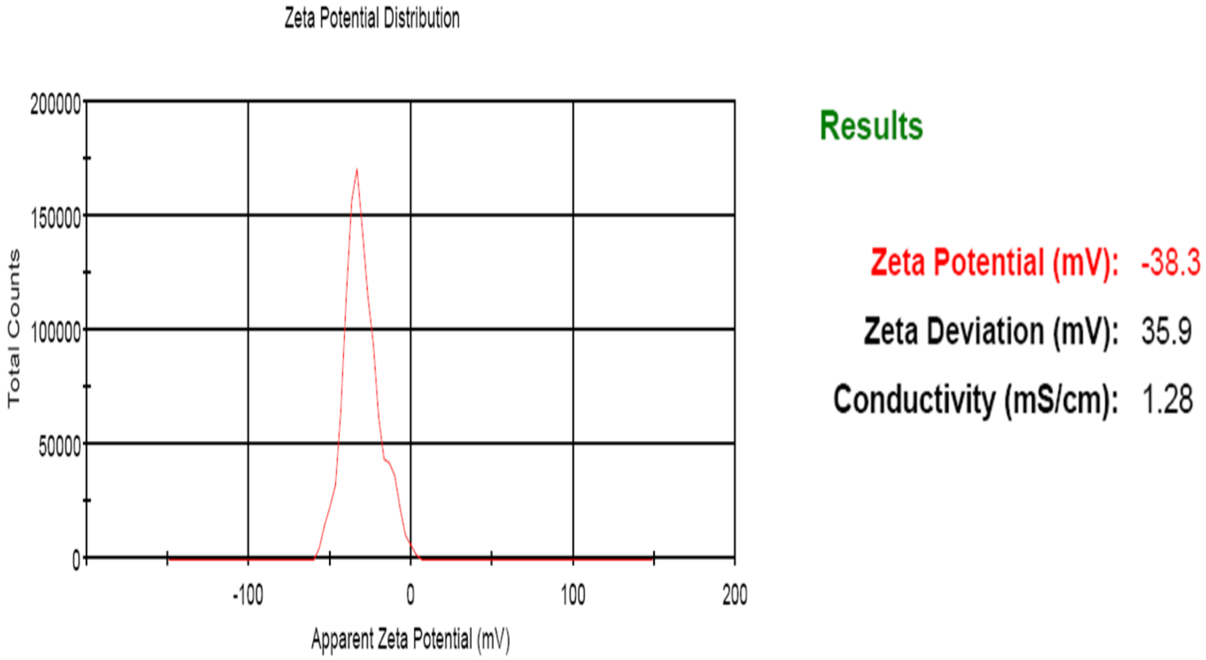
PdI: 0.152

Intercept: 0.947

Result quality : Good

	Size (d.nm):	% Intensity:	St Dev (d.n...
Peak 1:	33.32	100.0	12.50
Peak 2:	0.000	0.0	0.000
Peak 3:	0.000	0.0	0.000

Figure 4.19. Particle size of AuNPs by dynamic light scattering



Results

Zeta Potential (mV): -38.3

Zeta Deviation (mV): 35.9

Conductivity (mS/cm): 1.28

Figure 4.20. Zeta potential of AuNPs by dynamic light scattering

4.6.4 Gold nanoparticle-antibody conjugation

Formation of stable conjugation between AuNPs and anti-soy antibodies is another critical step for the development of LFA. In this study, a slight modification was made in a procedure prescribed by Lata *et al.,* (2015) was used for conjugation. Colloidal gold is negatively charged and since gold has natural affinity for protein, if conditions are made favorable, a stable conjugation is formed. pH of media is important and it has been advocated that pH of the medium should be just above pI of protein for effective conjugation (Chun, 2009). In this study, prepared AuNPs were initially buffer exchanged with borate buffer having pH 7.5 containing antibodies. The optimum concentration of antibody was 10 µl. To avoid occurrence of naked AuNPs, BSA is added to cap such AuNPs which would otherwise binds with nitrocellulose membrane or other antibodies on the test or control line. The conjugation was confirmed by running the lateral flow strip just with control line containing secondary antibodies. The appearance of red line indicated successful conjugation.

4.6.5 Principle of lateral flow strip and test determination

Usually in LFA, competitive format is used when testing for small molecules with single antigenic determinants, which cannot instantaneously bind to two antibodies. In competitive design, soymilk (antigen) is coated over test line. The assay is based on competitive reaction concept wherein free analyte in sample and soymilk coated at test line compete for limited amount of colloidal gold-labeled anti-soy antibody. If analyte is absent in the sample or below detection limit, the soymilk coated at test line would bind gold labeled anti-soy antibodies and thus accumulation of gold will create a red line. The intensity of the red color at test line is inversely proportional to the concentration of target analyte in the sample i.e. the more analyte in the test sample, the weaker the test signal and *vice-versa*. If the soymilk concentration in the sample is above a detection limit, whole of colloidal gold-labeled anti-soy antibody would bind to sample soymilk and thus would not be available for soymilk at test line and thus no line is formed. In such type of competitive formats of lateral flow assay, the amount of gold labeled anti-soy antibody deposited at conjugation pad is very crucial and should be same in all strips, at least in a batch. In this study, it was observed that 7 µl of gold labeled anti-soy antibody conjugate was sufficient and throughout the study, the similar conditions were maintained. Time taken for analysis is 5 min and test and control lines were stable for several months.

4.6.6 Detection of soymilk using AuNPs conjugated with antibody by lateral flow strip

Lateral flow strip is based on antigen antibody interaction. AuNPs coated with antibody (anti-soy) were used for detection of soymilk by LFA. The designing of lateral flow system comprises of sample pad, glass fibre (conjugate pad), nitrocellulose membrane and adsorbent pad placed in sequence on backing card. Two lines were drawn on the nitrocellulose membrane with the help of lateral flow printer. The test line consisted of soymilk and the control line which consisted of species specific (secondary) antibody. The nitrocellulose membrane was allowed to dry at room temperature for 30 min. Since antibodies are protein, these antibodies will bind to nitrocellulose membrane by various non-covalent forces. On the conjugation pad, AuNPs conjugated with anti-soy antibody were deposited and allowed to dry at room temperature for 10 min. As sample is placed on the sample pad of prepared strip, AuNPs move through capillary action across the membrane. At conjugation pad, some of nanoparticles get interacted and leave behind visible band. When test sample is positive only one line will appear i.e. control line and in absence of analyte two lines will appear i.e. both test and control line. In case of positive test sample, there is competition between soymilk on test line and soymilk present in sample. The AuNPs coated with antibody combine with soymilk in sample and development of test line will be absent while the same AuNPs will interact with secondary antibody and development of control line will occur. When test sample is negative then AuNPs coated with antibody interact with soymilk on test line producing a red colored line and the remaining AuNPs will interact with secondary antibody on the control line leading to the development of red line. Since AuNPs are red in color their accumulation at test or control line can be easily visualized by naked eye. The appearance of control line indicates the proper working of strip, while the absence of control line indicated the failure of the test. Results of Lateral flow strip can be obtained within 5 minutes. LOD in borate buffer was found to be 1.25% (Figure 4.21) and in skim milk 1.5% (Figure 4.22).

During the study, a difficulty was observed in the application of the developed lateral flow strip for the detection of soymilk in whole milk. It was perceived that as the viscosity of the whole milk was more, there was hindrance in the flow of whole milk through nitrocellulose membrane. To overcome this, milk was mixed with EDTA and then applied on the lateral flow strip. As the presence of EDTA in milk has disrupted the casein micelle (due to chelation of calcium in casein micelle), the viscosity of the EDTA added milk decreased vis-à-vis whole milk. The working of the strip was validated in such milk spiked with soymilk and results are presented in (Figure 4.23). The sensitivity of the method for the detection of soymilk in whole milk was 1.75%.

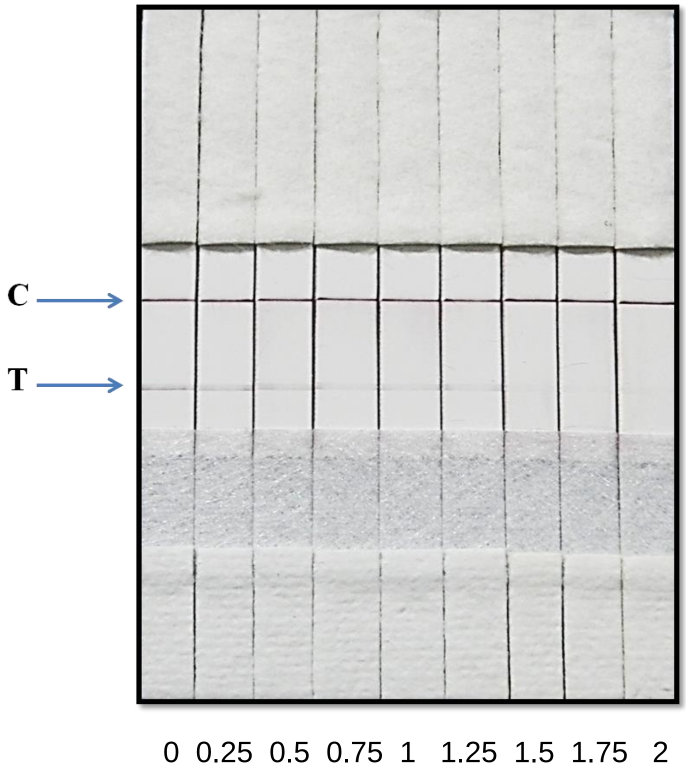


Figure 4.21. Detection of soymilk in Borate Buffer

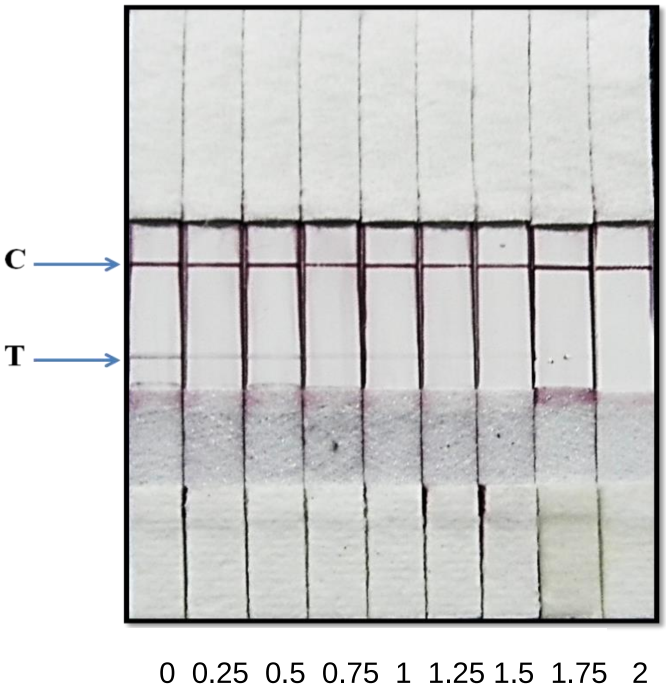


Figure 4.22. Detection of soymilk in Skim milk

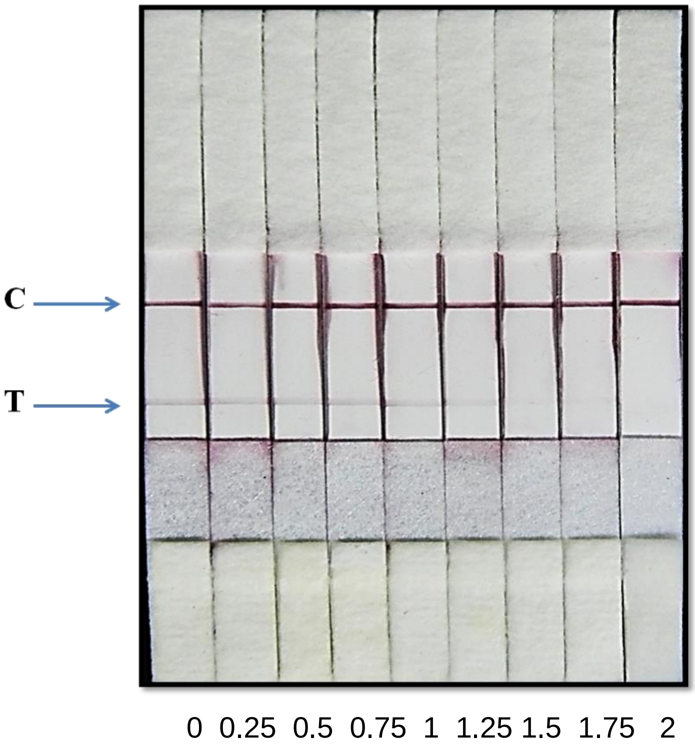


Figure 4.23. Detection of soymilk in whole milk

4.7 Alkaline Phosphatase detection strip

Alkaline phosphatase (ALP) is an enzyme naturally present in raw milk, which is used as an indicator for proper milk pasteurization. Non-pasteurized or raw milk contains ALP, which causes intra-abdominal bacterial infection after drinking the milk, whereas after pasteurization, ALP is denatured (Sharma *et al*., 2003). Therefore, milk industries test the milk after pasteurization using conventional methods such as colorimetric and fluorescence. Our attention was drawn to develop quick, simple and economical test using dry reagent strips for detection of ALP activity in milk. Two theories were adopted for the development of alkaline phosphatase detection strip in milk.

1) Using BCIP Red/NBT Solution A

2) Using phenolphthalein diphosphate tetrasodium salt

4.7.1 Working of alkaline phosphatase detection strip

4.7.1.1 Using BCIP Red/NBT Solution A

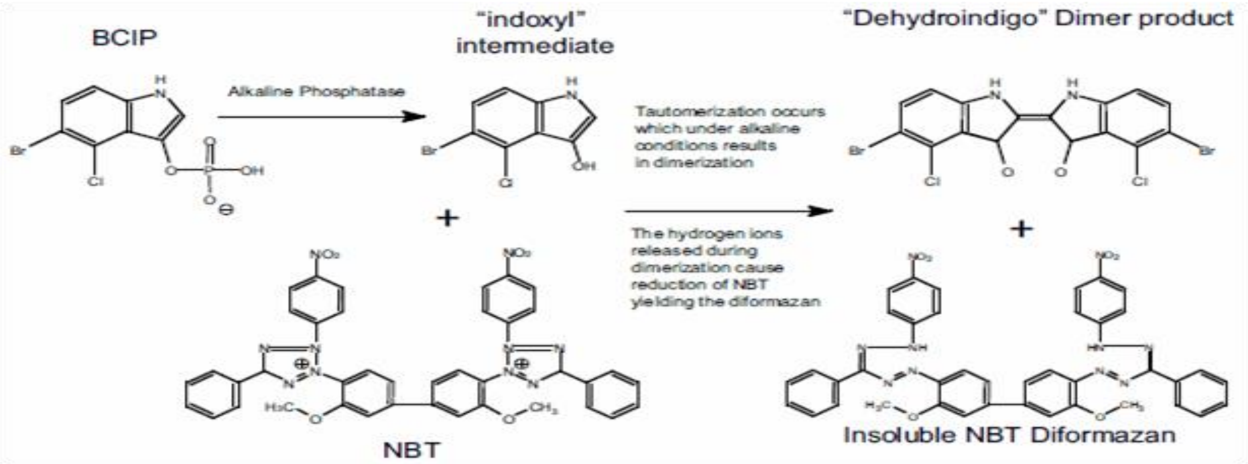


Figure 4.24. Reaction mechanism of BCIP/NBT red and Alkaline Phosphatase

BCIP Red/ NBT Solution A reacts with alkaline phosphatase to form blue to purple colored compound (Leary J.J *et al*., 1983; Trinh L.A. *et al*., 2007 and Schumacher *et al*., 2015) as depicted in (Figure 4.24). BCIP/NBT Solution A was immobilized on paper along with a basic buffer. The strip was applied to raw and pasteurized milk. Also the test was conducted in liquid phase for raw and pasteurized milk. Figure 4.25 (a) depicts the working of the hypothesis in liquid phase wherein clear distinction can be made between raw and pasteurized milk. In presence of alkaline phosphatase in raw milk, the solution turns blue while in heated milk, the colour of the solution remains off white. This difference in raw and heated milk, encouraged us to develop the paper based strip by immobilization of chemicals on paper strip. Figure 4.25(b) depicts the working of the hypothesis in dry phase. As no appreciable differentiation between raw and pasteurized milk could be made using dry chemistry so after a number of trials it was decided to stop the further work on the alkaline phosphatase strip using this concept.

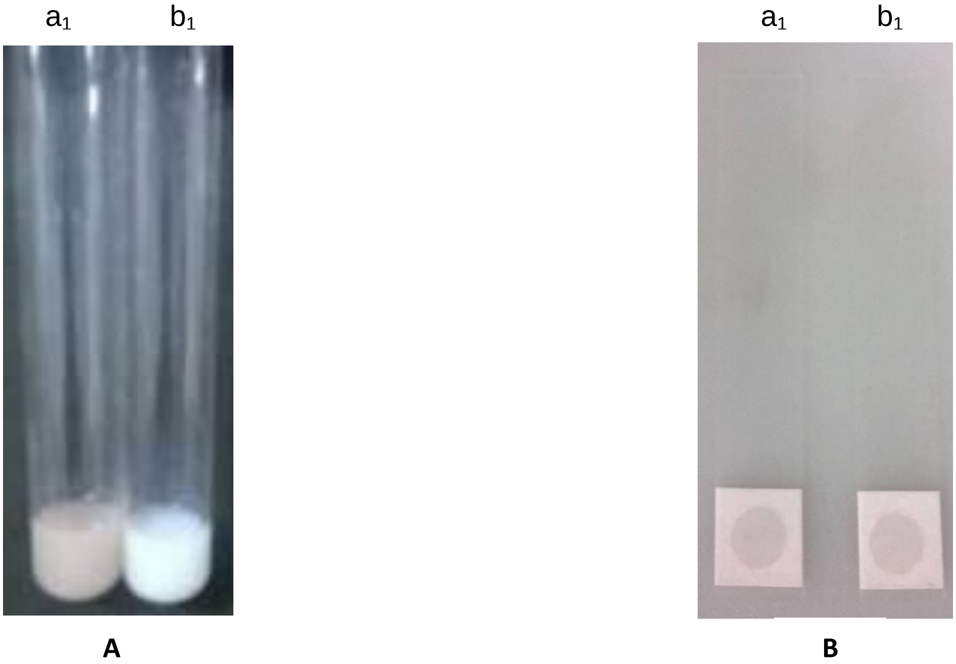
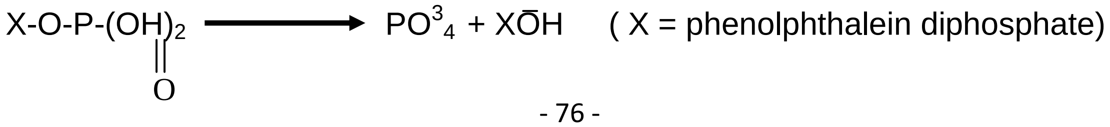


Figure 4.25 Reaction of alkaline phosphatase with colouring reagent in liquid and dry phase. (A). Differentiation between raw and pasteurized milk in liquid phase; (B). Differentiation between raw and pasteurized milk using dry reagent chemistry (a_1-raw milk, b_1-pasteurized milk)

4.7.1.2 Using phenolphthalein diphosphate tetrasodium salt

Phenolphthalein diphosphate reacts with alkaline phosphatase to form a pink colored compound in alkaline conditions (Huggins and Talalay, 1945; Klein *et al*., 1960; Rzhekhina, 1962; Pauwels, 1964; Babson *et al*., 1966)



$$X\text{-}O\text{-}\underset{\underset{O}{\|}}{P}\text{-}(OH)_2 \longrightarrow PO^{3}_{4} + X\bar{O}H \quad (\text{ X = phenolphthalein diphosphate})$$

Phenolphthalein diphosphate was immobilized on paper with a basic buffer. The strip was applied to raw and pasteurized milk. Also the test was conducted in liquid phase for raw and pasteurized milk. Figure 4.26 (a) depicts the working of the hypothesis in liquid phase wherein clear distinction can be made between raw and pasteurized milk. In presence of alkaline phosphatase in raw milk, the solution turns pink while in heated milk, the color of the solution remains white. This difference in raw and heated milk, encouraged us to develop the paper based strip by immobilization of chemicals on paper strip. Figure 4.26(b) depicts the working of the hypothesis in dry phase. As no appreciable differentiation between raw and pasteurized milk could be made using dry chemistry so it was decided to stop the further work on the alkaline phosphatase strip using this theory.

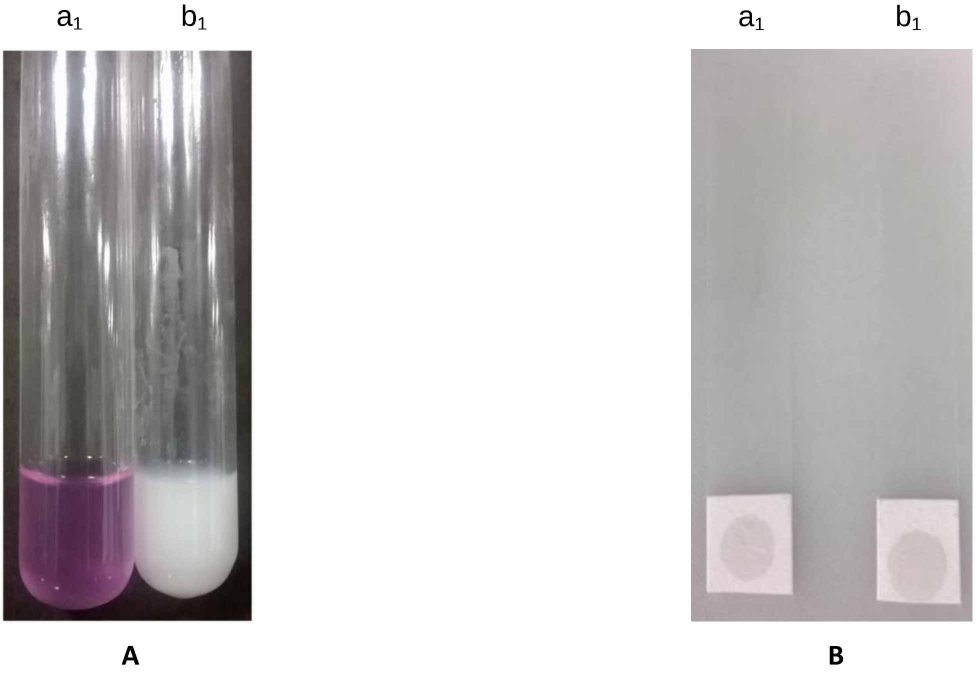


Figure 4.26 Reaction of alkaline phosphatase with colouring reagent in liquid and dry phase. (A). Differentiation between raw and pasteurized milk in liquid phase; (B). Differentiation between raw and pasteurized milk using dry reagent chemistry (a_1-raw milk, b_1-pasteurized milk)

4.8 Lactoperoxidase detection strip

Lactoperoxidase is one of the most heat stable enzymes in milk (Griffiths, 1986; Shakeel-ur-Rehman and Farkye, 2003) and its inactivation can be used as an index of the pasteurization efficiency of raw milk. LP is only partially inactivated by short pasteurization at 74 °C (Wolfson and Summer, 1993). Marks *et al*. (2001) confirmed that LP activity is not destroyed during normal pasteurization (63 °C for 30 min or 72 °C for 15 s), but it loses its activity when milk is heated at 80 °C for 4 s (Seifu *et al*., 2005). LP can also be used as a marker for subclinical mastitis in cows' milk (Isobe *et al*., 2011). LP and thiocyanate, together with hydrogen peroxide forms the LP system (LPS), which is considered to be the most significant microbial inhibitor and acts as cold pasteurization of milk where instant refrigeration of milk is not possible. LP also exhibits medicinal value (Tenovuo, 2002; Shimizu *et al*., 2011). Due to the important applications of LP, it is important to be able to detect its activity in milk and milk products during their processing, transportation and storage. Two theories were adopted for the development of lactoperoxidase detection strip in milk.

1) Using TMB liquid substrate for membranes
2) Using 4-AAP and Phenol

4.8.1 Working of Lactoperoxidase detection strip

4.8.1.1 Using TMB liquid substrate for membranes

Lactoperoxidase or peroxidase in the presence of H_2O_2 and TMB as a substrate produces blue color (EC Report, 1994; Shin *et al*., 2000; Che *et al.,* 2015). TMB along with citric acid was immobilized on paper. H_2O_2 and milk sample was applied on the strip and a change in color was observed. The strip was applied to raw and milk heated to 80°C. Also the test was conducted in liquid phase for raw and milk heated to 80°C. Figure 4.27 (a) depicts the working of the hypothesis in liquid phase wherein clear distinction can be made between raw and heated milk. In presence of lactoperoxidase in raw milk, the solution turns blue while in heated milk, the color of the solution remains white. This difference in raw and heated milk encouraged us to develop the paper based strip by immobilization of chemicals on paper strip. Figure 4.27 (b) depicts the working of the hypothesis in dry phase. As no appreciable differentiation between raw and milk heated to 80°C could be made using dry chemistry so it was decided to stop the further work on the lactoperoxidase strip using this theory.

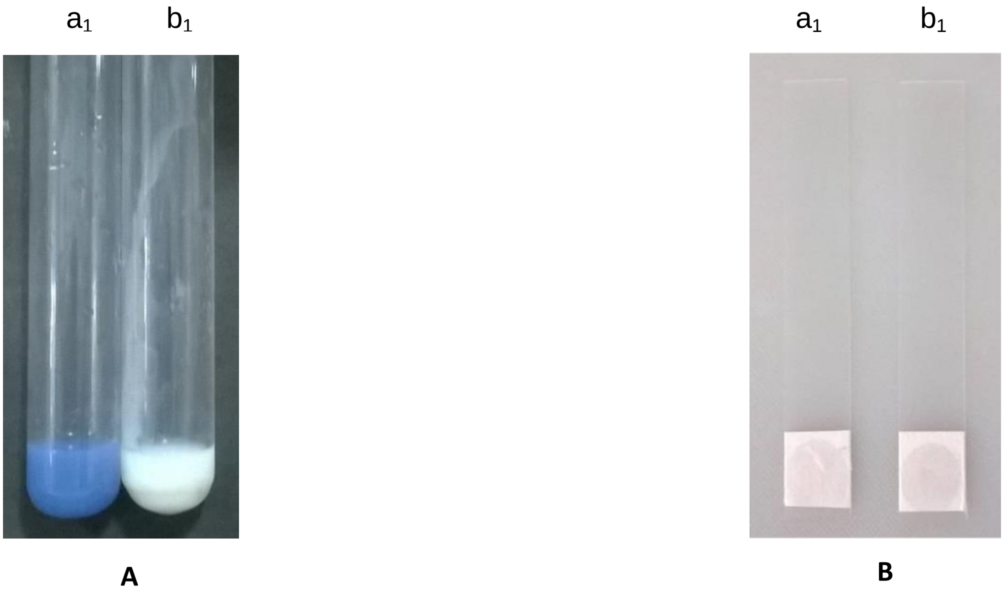


Figure 4.27 Reaction of lactoperoxidase with colouring reagent in liquid and dry phase. (A). Differentiation between raw and milk heated at 80°C in liquid phase; (B). Differentiation between raw and milk heated at 80°C using dry reagent chemistry (a_1- raw milk, b_1- milk heated at 80°C)

4.8.1.2 Using 4-AAP and Phenol

Lactoperoxidase in the presence of 4-AAP and Phenol produces pink color (Sharma and Rajput. 2014). 4-AAP, phenol, Tris-HCl buffer was immobilized on paper. The strip was applied to raw and milk heated to 80°C. Also the test was conducted in liquid phase for raw and milk heated to 80°C. Figure 4.28 (a) depicts the working of the hypothesis in liquid phase wherein clear distinction can be made between raw and heated milk. In presence of lactoperoxidase in raw milk, the solution turns pink while in heated milk, the color of the solution remains white. This difference in raw and heated milk encouraged us to develop the paper based strip by immobilization of chemicals on paper strip. Figure 4.28 (b) depicts the working of the hypothesis in dry phase. As no appreciable differentiation between raw and milk heated to 80°C could be made using dry chemistry so it was decided to stop the further work on the lactoperoxidase strip using this theory.

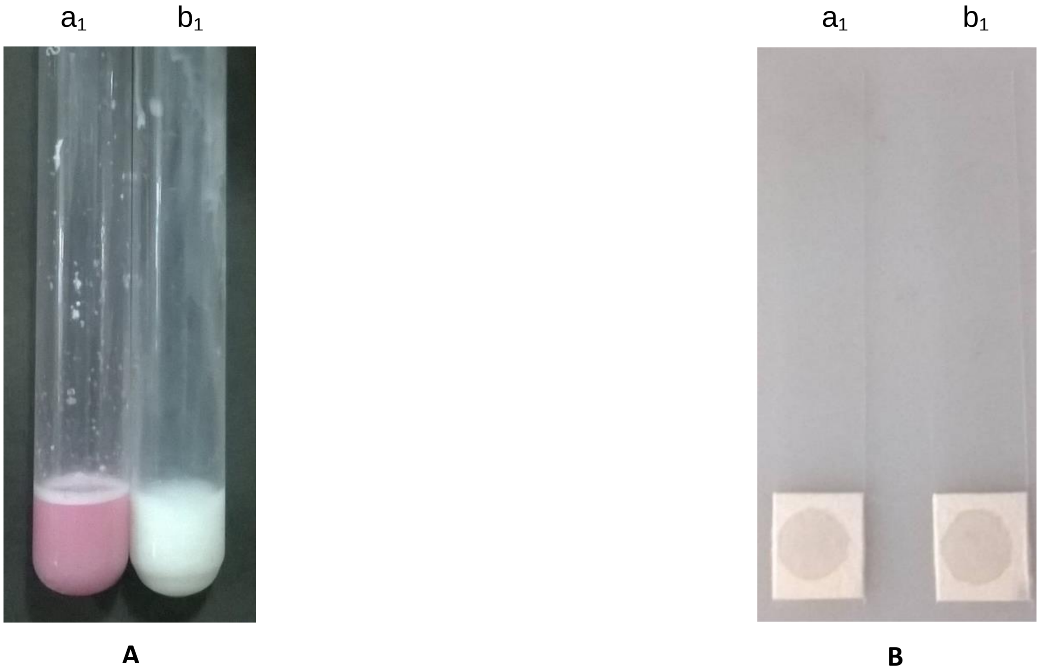


Figure 4.28(a). Reaction of lactoperoxidase with colouring reagent in liquid and dry phase. (A). Differentiation between raw and milk heated at 80°C in liquid phase; (B). Differentiation between raw and milk heated at 80°C using dry reagent chemistry (a_1- raw milk, b_1- milk heated at 80°C)

4.9 Validation of paper based strips in milk products (*Khoa, Dahi*)

In the earlier work done at our lab, two paper based strip were developed for rapid detection of glucose and maltodextrin in milk. The limit of detection of glucose and maltodextrin detection strip in milk were 0.03 and 0.05%, respectively (Sharma *et al*., 2016a, b). The sucrose detection strip was developed for rapid detection of sucrose in milk in the present study with limit of detection 0.1%. As there are reports of adulteration of milk products viz., *khoa* and *dahi* with glucose, sucrose and maltodextrin, a study was planned to check the efficacy of the developed strip for detection of these adulterants in milk products.

4.9.1 Validation of paper based strips in *Khoa*

Khoa was prepared as suggested by De (1991). The paper based strips were validated for the detection of adulterants like glucose, sucrose and maltodextrin.

4.9.1.1 Validation of glucose strips in *Khoa*

Milk was spiked at two levels i.e. 0.5 and 1% levels of glucose and the prepared *Khoa* was applied to the strip after making its 5% suspension in water. Glucose was detected at both the levels. Figure 4.29 (a) shows the detection of glucose at 0.5% level and Figure 4.29 (b) shows the detection of glucose at 1% level.

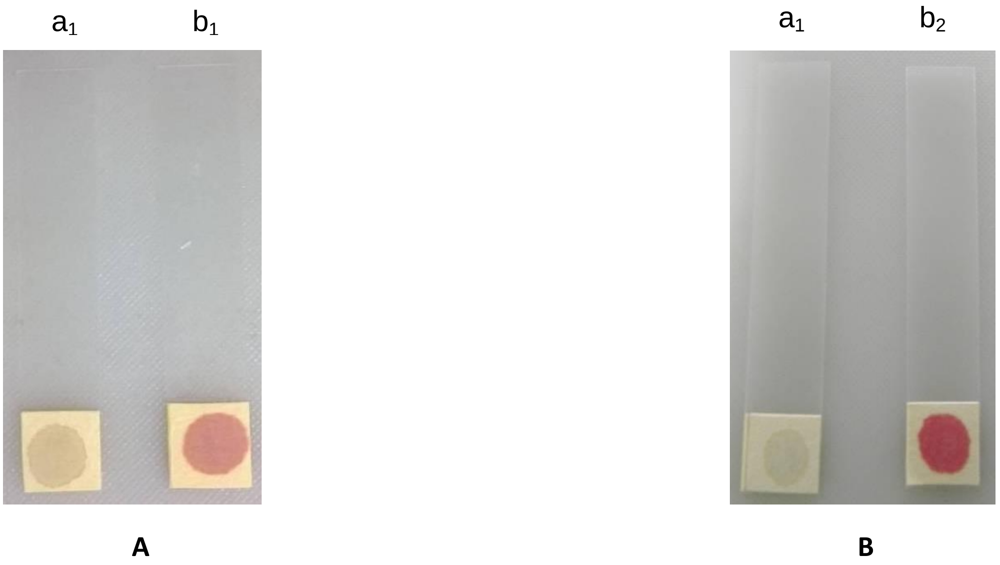


Figure 4.29. Detection of glucose in khoa. (A). Detection of glucose at 0.5% level; (B). Detection of glucose at 1% level (a_1- pure *khoa*, b_1 - *khoa* prepared from milk containing 0.5% glucose, b_2- *khoa* prepared from milk containing 1% glucose)

4.9.1.2 Validation of sucrose strips in *Khoa*

Milk was spiked at two levels i.e. 0.5 and 1% levels of sucrose and the prepared *Khoa* was applied to the strip after making its 5% suspension in water. Sucrose was detected at both the levels. Figure 4.30 (a) shows the detection of sucrose at 0.5% level and Figure 4.27 (b) shows the detection of sucrose at 1% level.

Figure 4.30. Detection of sucrose in khoa. (A). Detection of sucrose at 0.5% level; (B). Detection of sucrose at 1% level (a_1- pure *khoa*, b_1 - *khoa* prepared from milk containing 0.5% sucrose, b_2- *khoa* prepared from milk containing 1% glucose)

4.9.1.3 Validation of maltodextrin strips in *Khoa*

Milk was spiked at two levels i.e. 0.5 and 1% levels of maltodextrin and the prepared *Khoa* was applied to the strip after making its 5% suspension in water. Maltodextrin was detected at both the levels. Figure 4.31 (a) shows the detection of maltodextrin at 0.5% level and Figure 4.31 (b) shows the detection of maltodextrin at 1% level.

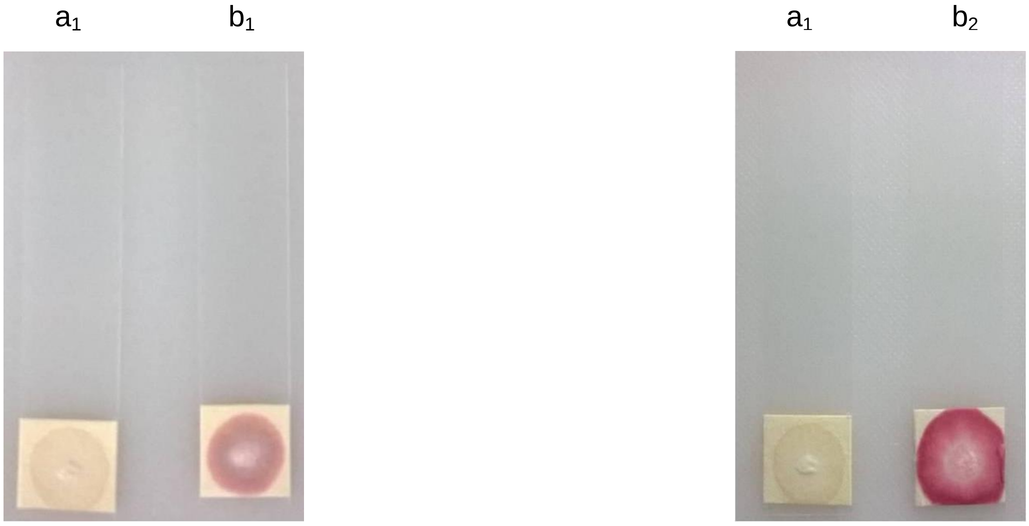
Figure 4.31 Detection of maltodextrin in khoa. (A). Detection of maltodextrin at 0.5% level; (B). Detection of maltodextrin at 1% level (a_1- pure *khoa*, b_1 - *khoa* prepared from milk containing 0.5% maltodextrin, b_2- *khoa* prepared from milk containing 1% maltodextrin)

4.9.2 Validation of paper based strips in *Dahi*

Dahi was prepared as suggested by De (1991) using culture NCDC 167. The paper based strips were validated for the detection of adulterants like glucose, sucrose and maltodextrin.

4.9.2.1 Validation of glucose strips in *Dahi*

Milk was spiked at two levels i.e. 0.5 and 1% levels of glucose and the prepared *Dahi* was applied to the strip after mixing the curd properly. Glucose was detected at both the levels. Figure 4.31 (a) shows the detection of glucose at 0.5% level and Figure 4.32 (b) shows the detection of glucose at 1% level.



A **B**

Figure 4.32 Detection of glucose in *Dahi*. (A). Detection of glucose at 0.5% level; (B). Detection of glucose at 1% level (a_1- pure *Dahi*, b_1 - *Dahi* prepared from milk containing 0.5% glucose, b_2- *Dahi* prepared from milk containing 1% glucose)

4.9.2.2 Validation of sucrose strips in *Dahi*

Milk was spiked at two levels i.e. 0.5 and 1% levels of sucrose and the prepared *Dahi* was applied to the strip after mixing the curd properly. Sucrose was detected at both the levels. Figure 4.33 (a) shows the detection of sucrose at 0.5% level and Figure 4.33 (b) shows the detection of sucrose at 1% level.

Figure 4.33 Detection of sucrose in *Dahi*. (A). Detection of sucrose at 0.5% level; (B). Detection of sucrose at 1% level (a_1- pure *Dahi*, b_1 - *Dahi* prepared from milk containing 0.5% sucrose, b_2- *Dahi* prepared from milk containing 1% sucrose)

4.9.2.3 Validation of maltodextrin strips in *Dahi*

Milk was spiked at two levels i.e. 0.5 and 1% levels of maltodextrin and the prepared *Dahi* was applied to the strip after mixing the curd properly. Maltodextrin was detected at both the levels. Figure 4.34 (a) shows the detection of maltodextrin at 0.5% level and Figure 4.34 (b) shows the detection of maltodextrin at 1% level.

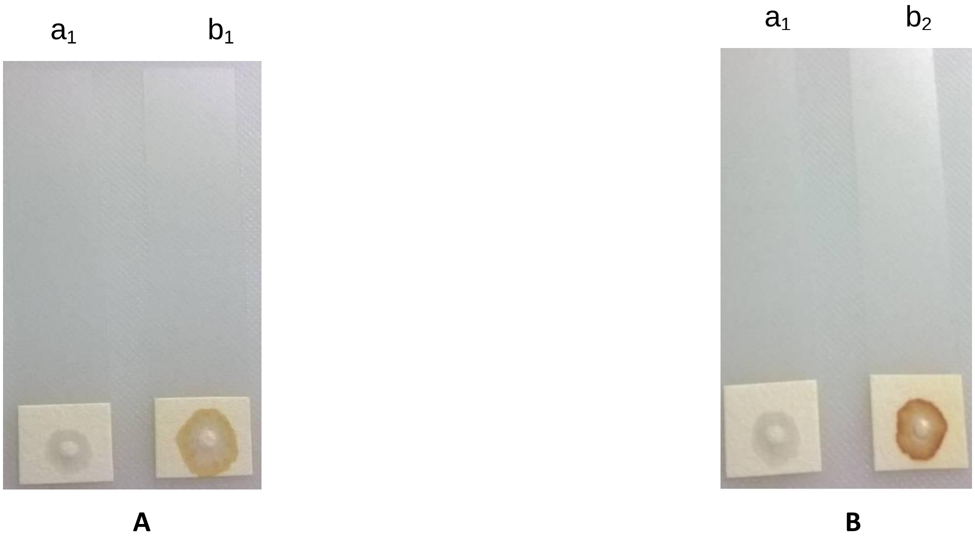


Figure 4.34 Detection of maltodextrin in *Dahi*. (A). Detection of maltodextrin at 0.5% level; (B). Detection of maltodextrin at 1% level (a_1- pure *Dahi*, b_1 - *Dahi* prepared from milk containing 0.5% maltodextrin, b_2- *Dahi* prepared from milk containing 1% maltodextrin)

4.10 Easy handling of strip at end point users

The strips were made in such a way so that it can be used easily at the end point users. To achieve it the cut strips were pasted with the help of a non reactive adhesive on an inert paper (which was specific for a certain kind of strip). Then it was cut into suitable size so that it can be used easily. Figure 4.35 shows the steps to be followed for making strips easy to handle.

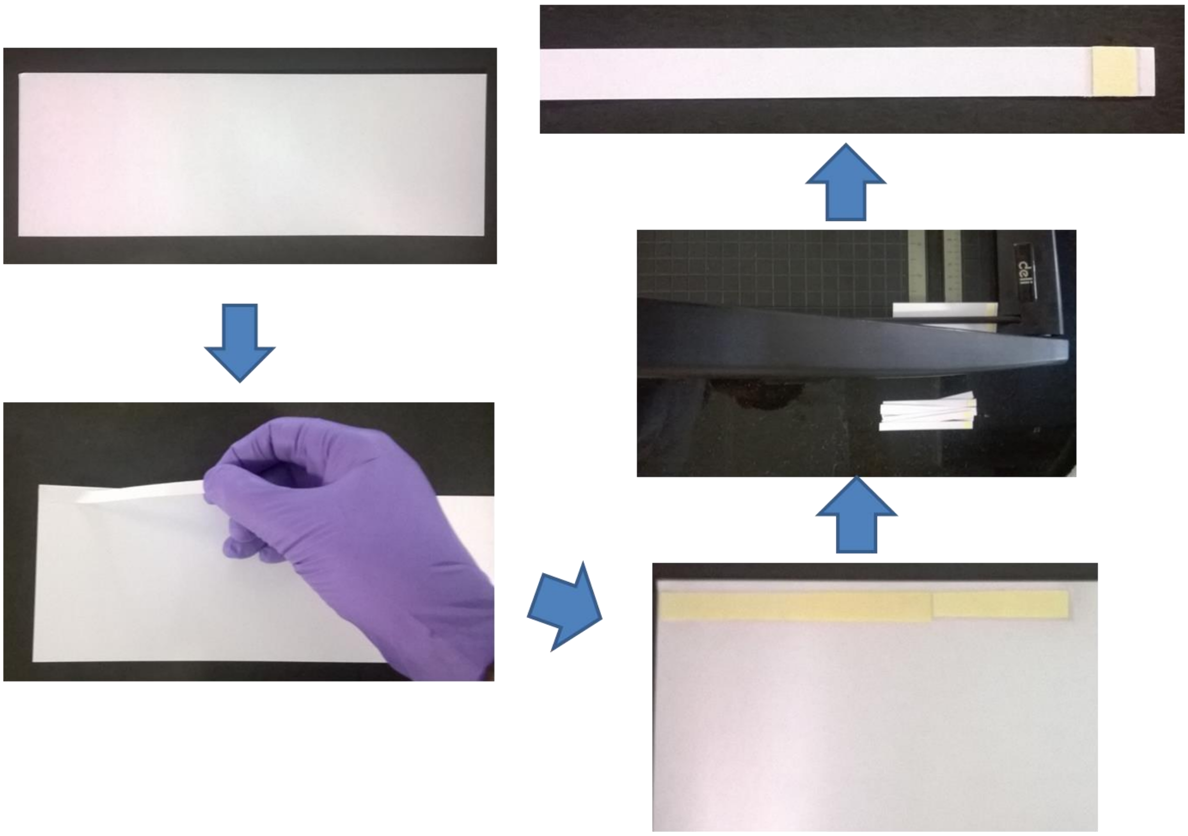


Figure 4.35. Steps followed for making easy to handle paper strips for detection of various adulterants in milk

CHAPTER – 5

Summary and Conclusion

5. SUMMARY AND CONCLUSION

- The new qualitative strip based methods for detection of neutralizers, urea, hydrogen peroxide and sucrose in milk have been developed. These test strips are based on the principles of dry reagent chemistry. Except the neutralizer detection strip, other three strips were based on multi-chromogens.
- The working of neutralizer detection strip is based on the change in the pH of milk upon neutralization. The over neutralized milk samples were detected by visualization of developed green color on the strip while in the case of pure milk sample the color of the strip remained yellow. The strip can detect 0.03% NaOH, 0.05% Na_2CO_3, 0.1% $NaHCO_3$ in milk. The developed strip was found to be stable upto 8 months at room temperature.
- The urea detection strip involved a series of reactions involving two chromogens. The milk samples containing added urea could be detected within a period of 3 minutes by appearance of red color, while in case of pure milk the strip remained orange in color. The strip was able to detect 0.08% of total urea (natural+added) in milk. The strip was found to be in a working condition for a period of five months at refrigeration temperature (4°C).
- The sucrose detection strip was developed on the principle that sucrose in the presence of invertase gets hydrolyzed to glucose and fructose. Glucose in turn is oxidized by glucose oxidase into hydrogen peroxide which in the presence of peroxidase and two chromogens produces a red colored compound within 3 minutes. The limit of detection for the developed strip was 0.1% sucrose. The shelf life of the strip was found to be 1 month at refrigeration temperature (4°C).
- The principle based for the detection of hydrogen peroxide is that potassium iodide produces a brown colored compound on reacting with hydrogen peroxide in presence of peroxidase. The adulterated milk was confirmed by the development of brown color. The limit of detection of the

strip was found to be 0.001%. The strip was found to be stable for a period of 5 months at refrigeration temperature (4°C).

- In terms of sensitivity, the three developed strips viz., detection of neutralizers, added urea and hydrogen peroxide in milk were found more sensitive vis-à-vis existing wet chemical methods. The developed strip for rapid detection of sucrose in milk was as sensitive as wet chemical method.
- An attempt was made so that the strips were easy to handle at the end user’s level. The active part of the strip was pasted on an inert paper using a non-reactive adhesive. The paper used in case of neutralizer and urea strips was different from those used for sucrose and hydrogen peroxide. The strip was observed to be stable over the entire period of storage for specific strip and its sensitivity was not affected at all.
- An attempt was made for the development of strips for the detection of detergent, alkaline phosphatase and lactoperoxidase. But the proposed hypothesis for the strips did not worked well in dry phase chemistry. So the work for the development of these strips was abandoned.
- The strips were also validated for the detection of various adulterants (glucose, sucrose and maltodextrin) in milk products (*Khoa* and *Dahi*).
- The strips were able to detect glucose, sucrose and maltodextrin at 0.5 & 1% level in both *Khoa* and *Dahi* respectively.
- A lateral flow based strip was developed for the detection of soymilk in milk using competitive format. The method involved conjugation of laboratory prepared gold nanoparticles with commercially available anti-soya antibody. The test line of lateral flow strip consisted of laboratory made soymilk while the control line of lateral flow strip consisted of secondary antibody (raised in goat against rabbit). The assay was validated with samples of soymilk in borate buffer, skim milk and whole milk and involved application of the samples to the sample pad of the lateral flow strip followed by visualization of appearance of one or two red lines within 5 minute on the nitrocellulose membrane.
- The detection limit of the soymilk was 1.25%, 1.5% and 1.75% in borate buffer, skim milk and whole milk, respectively.

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