DEVELOPMENT AND EVALUATION OF VITAMIN A AND IRON FORTIFIED MILK

THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENT
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
DAIRY CHEMISTRY

BY
BHAWANA
M.Sc. (Food Technology)

DAIRY CHEMISTRY DIVISION
NATIONAL DAIRY RESEARCH INSTITUTE
(I.C.A.R.)
KARNAL–132001 (HARYANA), INDIA
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Approved by:

EXTERNAL EXAMINER
01.10.12

(Dr. SUMIT ARORA)
MAJOR ADVISOR AND CHAIRMAN
(GUIDE)

MEMBERS, ADVISORY COMMITTEE

Dr. B. K. WADHWA
PRINCIPAL SCIENTIST, DC DIVISION
BKWadhwa

Dr. VIVEK SHARMA
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SENIOR SCIENTIST, DT DIVISION
AK Singh

Dr. NEELAM KEWALRAMANI
PRINCIPAL SCIENTIST, DCN DIVISION
1/10/12

DAIRY CHEMISTRY DIVISION
NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
(Indian Council of Agricultural Research)
KARNAL – 132001 (HARYANA), INDIA

Dr. SUMIT ARORA
SENIOR SCIENTIST

CERTIFICATE

This is to certify that the thesis entitled "**DEVELOPMENT AND EVALUATION OF VITAMIN A AND IRON FORTIFIED MILK**" submitted by **Ms. BHAWANA** in partial fulfilment of the requirement for award of the degree of **DOCTOR OF PHILOSOPHY** in **DAIRY CHEMISTRY** of the **NATIONAL DAIRY RESEARCH INSTITUTE (DEEMED UNIVERSITY)**, KARNAL (HARYANA) is a bonafide research work carried out by her under my supervision and guidance. The work embodied in this thesis is original and no part has been submitted in part or full for the award of any diploma or degree of this or any other university.

Date: 6/10/12

(Dr. SUMIT ARORA)
MAJOR ADVISOR AND CHAIRMAN
(GUIDE)

Website: http://ndri.res.in, GRAMS: DAIRY RESEARCH
FAX: 091-184 2250042, TELEX: 0396-204, PHONE: 091-184-2259156 (O)

Dedicated to
my loving
maa

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Bhawana

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(Bhawana)

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List of Abbreviations

$	Dollar
Å	Angstrom
cfu	Colony forming units
cP	Centipoise
d	Day
Da	Dalton
ft-c	Foot candle
g	Gram
hr	Hour
IU	International unit
k	Kilo
kg	Kilogram
L	Litre
M	Molar
mg	Milligram
ml	Millilitre
mM	Millimoles
mm	Millimetre
MRS	deMan-Rogosa-Sharpe
nm	Nanometer
°C	Degree Celsius
OD	Optical density
pH	Negative log of the hydrogen ion concentration
ppm	Parts per million
rcf	Relative centrifugal force
RE	Retinol equivalent
rpm	Revolutions per minute
U	Unit
y	Years
R/I	Retention/intake
Asp	Aspartic acid
C_{18}	Octadecyl carbon chain
CO_2	Carbon dioxide
Fe^{2+}	Ferrous ion
Fe^{3+}	Ferric ion
FeAC	Ferric ammonium citrate
$FeCl_2$	Ferrous chloride
$FeCl_3$	Ferric chloride

Fe-CN	Iron-casein complex
$FeSO_4$	Ferrous sulphate
Fe-WP	Iron-whey protein complex
HMF	5-hydroxy methyl furfural
IAFL	Iron+vitamin A fortified milk lyophilates
IFL	Iron fortified milk lyophilates
$K_2Cr_2O_7$	Potassium dichromate
KI	Potassium iodide
Lf	Lactoferrin
$Na_2S_2O_3$	Sodium thiosulphate
NaCl	Sodium chloride
NAD^+	Nicotinamide-adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide - hydrogen (reduced)
$NaHSO_4$	Sodium hydrogen sulphate
O_2	Oxygen
PGMS	Polyglycerol monostearate
Pi	Inorganic phosphate
Pser	Phosphoserine
RP	Retinyl palmitate
SEM	Standard error mean
SNF	Solid-not-fat
SDS	Sodium dodecyl sulphate
TBA	2-thiobarbituric acid
TEMED	N, N, N’ N’ tetramethylenediamine
TMB	Tetramethylbenzidine
Tween 60	Polyoxyethylene glycol sorbitan monostearate
Tween 80	Polyoxyethylene glycol sorbitan monooleate
UFL	Unfortified milk lyophilates
VA	Vitamin A acetate
WPI	Whey protein isolate
HCT	Heat coagulation time
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidise
IAEC	Institutional Animal Ethics Committee
ICMR	Indian Council of Medical Research
ICN	International Conference on Nutrition
IDA	Iron deficiency anaemia
IDD	Iodine deficiency disorder
INACG	International Nutritional Anaemia Consultative Group
NCDC	National Collection of Dairy Cultures
NFHS	National Family Health Survey

NGCP	National Goiter Control Program
NHD	Nutrition for Health and Development
NHRP	National Health Research Policy
NNMB	National Nutrition Monitoring Board
NNP	National Nutrition Policy
NPU	Net protein utilisation
NUT	Nutrition for health and development
PAGE	Polyacrylamide gel electrophoresis
PET	Polyethylene terephthalate
PFA	Prevention of Food Adulteration Act
RCT	Rennet coagulation time
RDA	Recommended dietary allowance
RNA	Ribonucleic acid
SDE	Sustainable development and healthy environments
TIBC	Total iron binding capacity
TLC	Thin-layer chromatography
TU	Tolerable upper intake levels
TX	Transferrin
UHT	Ultrahigh temperature
UIBC	Unsaturated iron binding capacity
USA	United States of America
USFDA	United States Food and Drug Administration
USPHS	United States Public and Health Service
UV	Ultra-violet
VAD	Vitamin A deficiency
WHC	Water holding capacity
WHO	World Health Organisation

सारांश

दूध प्रसंस्कृत, व्यापक रूप से वितरित तथा समाज के वर्गों द्वारा नियमित रूप से उपभुक्त किया जाता है | दूध लौह का एक अपर्याप्त (०.२ मी. ग्रा./कि. ग्रा.) स्त्रोत है तथा दूध से वसा के निकाले जाने पर विटामिन ए सहित कई वसा में घुलनशील विटामिन प्रथ्कारित हो जाते हैं | अतः, इस तरह के दूध में लौह एवं विटामिन ए को मिलाकर, इन पोषक तत्वों की अपूर्णता को दूर किया जा सकता है | ९ लौह लवणों तथा २ विटामिन ए एस्टर की छटनी के लिए संवेदी विश्लेषण, पी. एच., अम्लता तथा टीबीए मान के आधार पर टोंड दूध के पुष्टिकरण के लिए २ लौह लवणों (फेरिक पायेरोफोस्फेट घुलनशील, फेरस ग्लूकोनेट जल्योजित) एवं विटामिन ए एसीटेट का चयन किया गया | पुष्टिकरण का स्तर, एफ. पी. पी. घुलनशील/एफ. जी. जल्योजित के लिए १५, २० एवं २५ पी. पी. एम्. लौह तथा विटामिन ए एसीटेट के लिए २५०० आई. यू./ली. तय किया गया | फेरस सल्फेट के लाइपोसोम बनाने के लिए अंडा लेसीथिन का उपयोग भित्ति माल के रूप में किया गया, फिर उन्हें सुघन्धित दूध बनाने में प्रयोग किया गया | पुष्टिकृत दूध की सवेंदक स्वीकार्यता नियंत्रित दूध से तुल्य्नीय थी | यध्यपि, उच्च स्तर पर पुष्टिकरण से सुगंध में त्रुटी उत्पन्न होने की वजह से लाइपोसोम को और प्रयोग में नही लिया गया | पुष्टिकृत दूध (एफ. पी. पी. घुलनशील/एफ. जी. जल्योजित, २५ पी. पी. एम्. लौह) की उष्म स्थिरता नियंत्रित दूध से तुल्य्नीय थी | हालाँकि, पुष्टिकृत दूध के पी. एच., अम्लता तथा टीबीए मान में कुछ परिवर्तन था, परन्तु, इसका दूध पर कोई ठोस प्रभाव नहीं हुआ | विभिन्न प्रसंस्कृत प्रशोधन के उपरांत, लौह पुष्टिकृत दूध में विटामिन ए अधिक मात्रा में नष्ट हुआ | हालाँकि, अतिरिक्त विटामिन ए, मूल विटामिन ए की तुलना में अधिक स्थाई पाया गया | प्रसंस्कृत प्रशोधन का लौह की स्थिरता पर कोई प्रभाव नहीं देखा गया | विटामिन ए एवं लौह पुष्टिकरण का दही तनाव, रेंनेट जमाव अवधि, गाढ़ापन, रंग रूपरेखा तथा अल्कोहल स्थिरता पर प्रभाव अर्थपूर्ण नहीं था | भण्डारण के दौरान, पुष्टिकृत दूध की जारणकारी स्थिरता नियंत्रित दूध की तुलना में कम थी | अतिरिक्त लौह का मुख्य हिस्सा मट्ठे की तुलना में केजीन से बाध्य था | दूध के अतिशय अपकेंद्रीकरण से यह ज्ञात हुआ की ८८-९० प्रतिशत दोनों, मूल एवं अतिरिक्त लौह दूध की कोलोइडल प्रावस्था में कायम है | दूध प्रोटीन के वैद्युतकणसंचलन व्यवहार पर पुष्टिकरण का कोई असर नहीं हुआ | उबले हुए, विसंक्रमित दूध में मेलार्ड ब्राउनइंग पाश्चरीकृत दूध की तुलना में अधिक थी | लौह पुष्टिकृत दूध में एच. एम्. एफ. एवं फ्युरोसिन तत्व नियंत्रित दूध की तुलना में अधिक थे | नियंत्रित दूध और पुष्टिकृत दूध में वसा के स्तर से सवेंदक स्वीकार्यता, दही तनाव, रेंनेट जमाव अवधि तथा जारणकारी स्थिरता अर्थपूर्ण रूप से प्रभावित थे | पुष्टिकृत दूध से निर्मित दही और योगर्ट के सवेंदक, भौतिक/रासायनिक और सुक्षमजीव विज्ञानी गुण नियंत्रित दूध के गुणों से तुल्यानीय थे | अतिरिक्त पोषक तत्वों की इन विट्रो सुपाच्यता मूल पोषक तत्वों से तुअल्य्नीय है | पशु परिक्षण की द्रष्टि से एफ. पी. पी. घुलनशील को चुना गया, क्योंकि दूध की संरचना पर इसका प्रभाव एफ. जी. जल्योजित की तुलना में कम था | इन वीवो परिणाम से यह ज्ञात होता है की विटामिन ए+लौह पुष्टिकृत दूध के पोषण से पशु में लौह तथा विटामिन ए का स्तर में वृधि हुई | यह प्रभाव रक्ताल्पता पीड़ित चूहे में नियंत्रित चूहों की तुलना में सुस्पष्ठ है | अतः, दूध में पुष्टिकरण का सुनिणीर्त स्तर २५ पी पी एम् लौह (एफ. पी. पी. घुलनशील) तथा २५०० आई यू/ली. विटामिन ए (एसीटेट) निर्धारित किया गया | २५० मी. ली. पुष्टिकृत दूध ३०-३५ प्रतिशत लौह तथा विटामिन ए की आवश्यकता पूर्ति करने में सक्षम होगा |

ABSTRACT

Milk is centrally processed, widely distributed and regularly consumed by all sections of the society. Milk is a poor source of iron (0.2 mg/kg) and reduction in fat content of milk leads to removal of major portion of fat soluble vitamins including vitamin A. Fortification of such milk with iron and vitamin A can help to eliminate the deficiency of these nutrients in target population. Two iron salts (ferric pyrophosphate soluble and ferrous gluconate hydrate) and vitamin A acetate were selected after screening of nine iron salts and two vitamin A esters for fortification of toned milk (cow milk:buffalo milk 1:1) on the basis of sensory analysis, pH, acidity and TBA value. The level of fortification selected was 15, 20 and 25 ppm iron for FPP soluble/FG hydrate and 2500 IU/L for vitamin A acetate. Liposomes of ferrous sulphate were prepared using egg lecithin as wall material. Flavoured milk (fortified@5, 10 and 15 ppm iron) was prepared using liposomes and sensory acceptability of fortified milk (@5 ppm) was comparable to control. However, higher level of fortification led to pronounced flavour defects in milk, hence, liposomes were not considered for further experiments.

The fortified milk (FPP soluble and FG hydrate@25 ppm iron) had comparable heat stability with that of control milk. There were slight changes in pH, acidity and TBA value of fortified milk (vitamin A+iron), however, there was no detrimental effect on milk system. During different processing treatments, loss of vitamin A was more in case of iron fortified milk. However, added vitamin A was found to be more stable than native vitamin A. No effect of processing treatments was observed on the stability of iron in milk. There was non significant effect of vitamin A and iron fortification on curd tension, rennet coagulation time, viscosity, colour profile and alcohol stability of milk. During storage, fortified milk had lesser oxidative stability compared to control milk. Major portion of the added iron was bound to casein compared to whey. Ultracentrifugation of milk revealed that 88-90% of both natural and added iron was retained in colloidal phase of milk. There was no effect of fortification on electrophoretic behaviour of milk proteins. Maillard browning was more in boiled and sterilised milk compared to pasteurised milk; HMF and furosine content were higher in iron fortified milk compared to control milk. Sensory acceptability, curd tension, viscosity and oxidative stability of control and fortified milk were significantly affected by the fat content. Sensory, physico-chemical and microbiological properties of *dahi* and yoghurt prepared from fortified milks were comparable with their control counterparts. *In vitro* digestibility of the added nutrients was comparable to those inherently present in milk. FPP soluble was selected for conducting animal trials due to the fact that it caused lesser changes in milk system compared to FG hydrate. *In vivo* results revealed that vitamin A+iron fortified milk improved iron and vitamin A status of experimental animals; vitamin A enhanced the absorption of iron and vice versa (biological indices, haemoglobin repletion, plasma ferritin, plasma transferrin and liver status of vitamin A and iron). The effects were more pronounced in anaemic rats compared to control rats. The optimum fortification level in milk was 25 ppm iron (FPP soluble) and 2500 IU/L vitamin A acetate. A 250 ml serving of the fortified milk will provide ~30-35% of iron and vitamin A requirement on daily basis.

CHAPTER – 1

Introduction

1. Introduction

Humans need a wide range of nutrients to lead a healthy and active life. The required nutrients for different physiological groups can only be derived from a well balanced diet. Micronutrient malnutrition is a serious threat to the health and productivity of billions of people worldwide, even though it is largely preventable (Diosady and Rutkowski 2007). Micronutrient deficiencies represent a largely invisible but often devastating form of malnutrition. Known effects of micronutrient deficiencies include impaired physical growth and cognitive development among children, poor immune response, loss of energy and productivity. Globally, micronutrient deficiencies of greatest public health significance are those of iron, vitamin A and iodine (NHD/SDE/WHO 2000).

Iron deficiency has earned distinction as the most common nutritional deficiency in the world today (Navarrete *et al.* 2002; Horton and Ross 2003). In 2002, iron deficiency anaemia (IDA) was considered to be among the most important contributing factors to the global burden of disease. Iron deficiency anaemia (IDA) affects almost 2 billion people worldwide, producing physical and psychical diseases, reducing productivity and affecting the health budget (Lotfi *et al.* 1996). Iron deficiency is usually the result of insufficient dietary intake of iron, poor utilisation of iron from the ingested food or a combination of the two (Gaucheron 2000). Moreover, iron deficiency in terms of deficient iron stores in the body can exist without clinical anaemia.

Vitamin A deficiency (VAD), affecting more than 100 million children in 75 countries; can lead to partial or total blindness (Diosady and Rutkowski 2007). VAD is the second largest cause of global blindness next to cataracts. The major cause of VAD is inadequate dietary intake of the preformed retinol or precursors of vitamin A. Increased vitamin A requirement in certain physiological or pathological conditions, inadequate absorption or loss of intestinal contents in diarrhea are often contributory factors in establishing vitamin A deficiency. VAD is also associated with increased risk of infectious morbidity and mortality.

According to a World Bank publication, "Deficiencies of just vitamin A, iodine and iron. could waste as much as 5% of gross domestic product (GDP), however, addressing them comprehensively and sustainably would cost less than 0.3% of GDP” (McGuire and Galloway 1994).

The control of vitamin and mineral deficiencies is an essential part of the overall effort to fight hunger and malnutrition. Therefore, overcoming micronutrient malnutrition is a precondition for ensuring rapid and appropriate national development. Pharmaceutical supplementation, dietary diversification and food fortification are some useful strategies for the control of micronutrient malnutrition (Allen *et al.* 2006). Out of these, fortification of foods with vitamins and minerals can be an effective strategy to combat micronutrient deficiencies in developing countries. The choice of proper vehicle and the nutrients is a key to the effectiveness of fortification programmes.

Diet-related micronutrient deficiencies rarely occur in isolation; deficiencies of iodine and vitamin A or of iron and vitamin A or zinc are often observed in the same populations (Badham *et al.* 2007). Lack of vitamin A and iron in humans and experimental animals may result in anaemia. Therefore, combined supplementation of vitamin A and iron may have positive influence on health (Schultink and Gross 1996). Multiple fortification of foods is a possible way of addressing deficiencies of two or more micronutrients at the same time in a cost-effective manner (Darnton-Hill and Nalubola 2002).

Milk could be considered as an appropriate food vehicle for vitamin A and iron delivery to correct the dietary deficiency of these nutrients as it is centrally processed, widely distributed and regularly consumed by all sections of the society in predictable amounts.

Micronutrients added to milk can be destroyed during the normal thermal processing and storage of milk. To compensate for these losses, an appropriate overage of each micronutrient must be added during fortification. Vitamin and mineral fortification program requires periodic testing to ensure the desired amount of micronutrient in the final product prior to use. Bioavailability is a key consideration when developing strategies for preventing mineral deficiencies through improved dietary supply.

It is well known fact that the vitamin A content of milk supplied through dairies is reduced to a great extent due to removal of fat and during processing in manufacture of toned and double toned milks. As low fat dairy products became popular, reduction of vitamin A in these products has rendered a nutritional concern. Vitamin A content of whole milk is around 206 IU/100 g or 62 µg/100 g (Fox and McSweeney 1998), however, its content in milk supplied through dairies is reduced to a great extent due to removal of fat and during processing in manufacture of toned milk (60 IU/100 g or 18 µg/100 g) and double toned milk (36 IU/100 g or 11 µg/100g) (www.piramalhealthcare.com, accessed on September 19, 2011). Fortification of such milks with vitamin A is the only remedy to overcome the vitamin A deficiency of these milks. Recommended dietary allowance for vitamin A is 600 µg/day i.e. 2000 IU/day (ICMR 2009). Fortification of liquid milk with vitamin A (2000 IU/L) gave acceptable results (Bector and Rani 1998). Vitamin A addition to whole milk is optional, however, low fat and non-fat milk must be fortified so that each quart contains ≥2000 IU vitamin A (Code of Federal Regulations 1985).

Milk is relatively poor in iron (0.2-0.4 mg/L) (Flynn 1992). Direct addition of iron to milk or dairy products might be an effective mean of increasing the dietary intake of iron of general population. However, iron fortification is difficult in food processing due to its potential for oxidised off-flavour, colour change, sedimentation and metallic flavour, probably as a result of lipid peroxidation of milk fat. Microencapsulation which has the potential as a carrier in food system could be a good method for adding iron to dairy products (Kwak *et al.* 2003). Recommended dietary allowance (RDA) for iron is 17 mg/day for adult men and 21 mg/day for adult women (ICMR 2009).

Several physico-chemical changes may take place due to mineral and vitamin fortification of milk, which may be reflected in the form of changes in colour, sedimentation, flavour, viscosity and/or the functional properties of the product. Improved understanding of the interactions between added micronutrients and components of milk and the physical functionality of fortified milk may help to reduce the undesirable changes that may occur upon mineral and vitamin addition to milk.

In some countries, optional and mandatory fortifications are in place. However, fortification has still not gained a foothold in our country. Till date, work has been done on single micronutrient fortification of milk, however, very few studies have reported multiple fortification of milk. Therefore, research is needed to explore the feasibility of multiple micronutrient fortification of milk with vitamin A and iron and finding out the interactive role of the two-if any.

Hence, the present investigation entitled “Development and evaluation of vitamin A and iron fortified milk” was proposed with the view of fortifying milk with vitamin A and iron to eliminate the deficiencies of these micronutrients with the following objectives:

i. To identify the levels and types of vitamin A and iron compounds to be added to milk as fortificants.
ii. To develop a technology for fortification and evaluation of fortified milk for stability and physico-chemical parameters.
iii. Effect of fortification on sensory, physical and microbiological properties of *dahi* and yoghurt.
iv. To study the bioavailability of added micronutrients in the fortified milk.

CHAPTER –2

Review of Literature

2. Review of Literature

2.1 Status of micronutrient malnutrition

Micronutrient deficiencies such as iron deficiency anaemia (IDA), vitamin A deficiency (VAD) and iodine deficiency disorders (IDD) continue to be significant public health problems; affecting more than a third of the world's population (Lotfi *et al.* 1996). Apart from well established deficiency cases of iron, iodine and vitamin A; emerging evidences have been reported on low plasma levels of zinc, folic acid and vitamin D as well. Sporadic deficiencies related to vitamin B_1, B_2, B_{12} and evidences of increasing fracture risk in Indian population attributed to calcium and vitamin D deficiency have also been reported in recent past (NNMB 2003; Gragnolati *et al*. 2005; Marwaha *et al*. 2005; Sachan *et al*. 2005; Salvi and Damania 2005; Harinarayan *et al*. 2007; Pathak *et al*. 2007; Puri *et al*. 2008; Teotia and Teotia 2008; Yajnik *et al*. 2008; Krishnaveni *et al*. 2009; Babu and Calvo 2010; Bandgar and Shah 2010; Singh *et al.* 2010; Jain *et al*. 2011; Osei *et al*. 2010; Kapil and Jain 2011).

Iron deficiency has earned distinction as the most common nutritional deficiency in the world today. It affects more than 20% of world's population because iron imparts an important role in the formation of blood (Walter *et al*. 1998; Navarrete *et al*. 2002). Iron deficiency often leads to anaemia, defined as having a blood haemoglobin level below standard which is usually the result of insufficient dietary intake of iron, poor utilisation of iron from ingested food or a combination of the two (Gaucheron 2000). The incidence of iron deficiency anaemia (IDA) is high among infants, teenagers and women of child bearing age, in both developing and industrialised countries. About 3.5 billion persons in the developing world are affected by this nutritional disorder (ACC/SCN 2000). The prevalence of anaemia among pregnant women is 52% in the world and 76% in South Asia (WHO 2000). Global prevalence of anaemia is 47.4% in preschool aged children, 41.8% in pregnant women and 30.2% in non-pregnant women (Badham *et al*. 2007). In India, prevalence of anaemia is 79% in children of 6-35 months age, 55% in women of 15-49 years and 24% in men of 15-49 years of age (NNP 1993; NFHS 2007).

Vitamin A deficiency (VAD) exists in more than 60 countries, at a clinical or subclinical level. VAD is the second largest cause of global blindness next to cataracts. The major cause of vitamin A deficiency is inadequate dietary intake of the preformed retinol or precursors of vitamin A. Increased vitamin A requirement in certain physiological or pathological conditions, inadequate absorption or loss of intestinal contents in diarrhea are often contributory factors in establishing vitamin A deficiency. An estimated 250 million preschool children are vitamin A deficient and this deficiency is the leading cause of preventable blindness in children and increases the risk of disease and death from severe infections. In pregnant women, VAD causes night blindness and may increase the risk of maternal mortality. An estimated 250000 to 500000 vitamin A deficient children become blind every year, half of them dying within 12 months of losing their sight (Allen *et al*. 2006).

Vitamin A supplementation programme for children under five has been running for several decades. As per the National Family Health Survey III (2005-06), only 25% of the under-fives received a dose of vitamin A in the 6 months preceding the survey (NFHS 2007). Clinical symptoms of VAD such as night blindness are seen among women of reproductive age and in pregnant women (Gragnolati *et al*. 2005). This clearly shows that all age groups are at risk of vitamin A deficiency and there are no specific interventions to cover various population groups, specifically, the children above 5 years, pregnant women and women in post-partum phase.

2.2 Strategies for the control of micronutrient malnutrition

The control of vitamin and mineral deficiencies is an essential part of the overall effort to fight hunger and malnutrition. Pharmaceutical supplementation involves the administration of supplements to individuals diagnosed with a certain micronutrient deficiency; however, it fails owing to poor compliance, poor coverage, absence of commitment at the national and community levels and poorly designed communications (Gibson *et al.* 1998). The International Conference on Nutrition (ICN) held in Rome in 1992 emphasised the importance of food based activities in their plan of action geared at addressing the issue of micronutrient malnutrition (FAO/WHO 1992).

Micronutrient deficiencies can be prevented and even eliminated if optimal quantities of the micronutrients are consumed by populations on a regular basis. The 11th five year plan (2007-2012) report of the working group on integrating nutrition with health (www.motherchildnutrition.org/india/pdf/mcn-integrating-nutrition-with-health.pdf, accessed on December 06, 2011) and the Draft National Health Research Policy 2010 (NHRP 2010) identified micronutrient malnutrition as a major concern. The loss due to micronutrient deficiencies costs India 1% of its GDP which is ultimately a loss of Rs. 27720 crores per annum in terms of productivity, illness, increased health care costs and death (Kotecha 2008). “Providing micronutrients” has been recognised as having the best cost/benefit ratio to achieve a major impact in the developing world as stated by more than fifty economists in the Copenhagen Consensus, 2008 (www.copenhagenconsensus.com, accessed August 11, 2010). Food fortification, the addition of specific nutrients to food or water, has been shown to be an effective strategy to combat micronutrient malnutrition (Cook and Reusser 1983; Stekel *et al.* 1988).

2.3 Food fortification

Food fortification is the public health policy of adding micronutrients (essential trace elements and vitamins) to foodstuffs to ensure that minimum dietary requirements are met. Food fortification has been defined as the addition of one or more essential nutrients to a food, whether or not it is normally contained in the food, for the purpose of preventing or correcting a demonstrated deficiency of one or more nutrients in the population or specific population groups (FAO/WHO 1994). Food fortification differs from other programmes that it involves the addition of nutrients to foods in the respect that it is a nutritional intervention with a specifically defined target and fortified food products are expected to become a main source of the specific added nutrient. Consequently, food fortification is expected to help in preventing nutritional inadequacy in targeted populations in which a risk of nutrient deficiency has been identified. A realistic level of fortification is to provide the equivalent of 20-40% of the daily requirements of target nutrient through a single food item (Huma *et al*. 2007).

The public health benefits of fortification may either be demonstrable or indicated as potential or plausible by generally accepted scientific research, and include:

- Prevention or minimisation of the risk of occurrence of micronutrient deficiency in a population or specific population groups.
- Contribution to the correction of a demonstrated micronutrient deficiency in a population or specific population groups.
- Potential for improvement in nutritional status and dietary intakes that may be, or may become, suboptimal as a result of changes in dietary habits or lifestyles.
- Plausible beneficial effects of micronutrients consistent with maintaining or improving health (e.g. there is some evidence to suggest that a diet rich in selected antioxidants might help to prevent cancer and other diseases).

2.3.1 Types of fortification

Food fortification can take several forms. It is possible to fortify foods that are widely consumed by the general population (mass fortification); foods designed for specific population subgroups, such as complementary foods for young children or rations for displaced populations (targeted fortification); and/or to allow food manufacturers to voluntarily fortify foods available in the market place (market-driven fortification). Mass fortification is nearly always mandatory, while targeted fortification can be either mandatory or voluntary depending on the public health significance of the problem it is seeking to address. Market-driven fortification is always voluntary, however, it is governed by regulatory limits (Allen *et al*. 2006).

2.3.1.1 Mass fortification

Mass fortification is the term used to describe the addition of one or more micronutrients to foods commonly consumed by the general public, such as cereals, condiments and milk. It is usually instigated, mandated and regulated by the government sector. Mass fortification is generally the best option when the majority of the population has an unacceptable risk, in terms of public health, of being or becoming deficient in specific micronutrients. Mass food fortification is being promoted as the nutritional intervention to prevent the physiological and clinical consequences associated with micronutrient deficiencies and has the most favourable cost to benefit ratio. Although this statement may be true in many cases, it depends on the existence of several preconditions. These include:

- Accessibility of the food fortification vehicle by the target population.
- Adequate manufacturing and industrial settings which ensure cost effective production and supervision.
- Compatibility of fortificants (the source of micronutrients) with the nature and use of the food vehicle.
- Affordability of additional costs resulting from the fortification process for consumers and manufacturers.
- Confirmation of the quality of the product at the production site.
- Reliable enforcement actions by government authorities to assure compliance of standards and regulations.
- Legitimate and justifiable food labeling and nutrition and health claims that promote healthy practices.

  (www.a2zproject.org/pdf/Food-Fortification-Formulator.pdf, accessed on September 16, 2010)

2.3.1.2 Targeted fortification

In targeted food fortification programmes, foods aimed at specific subgroups of the population are fortified, thereby increasing the intake of that particular group rather than that of the population as a whole. Examples include complementary foods for infants and young children, foods developed for school feeding programmes, special biscuits for children and pregnant women and rations (blended foods) for emergency feeding and displaced persons. In some cases, such foods may be required to provide a substantial proportion of daily micronutrient requirements of the target group (Allen *et al*. 2006).

2.3.1.3 Market-driven fortification

The term “market-driven fortification” is applied to situations whereby a food manufacturer takes a business-oriented initiative to add specific amounts of one or more micronutrients to processed foods. Although voluntary, this type of food fortification usually takes place within government-set regulatory limits (Allen *et al*. 2006).

2.3.2 History of food fortification

Salt iodisation was introduced in the early 1920s in both the United States of America (Marine and Kimball 1920) and Switzerland (Burgi *et al.* 1990) and has expanded progressively all over the world to the extent that iodised salt is now used in most countries. Salt iodisation in India started with the National Goiter Control Program (NGCP) in 1962 and gained momentum in early 1980s. The remarkable increase in production of iodised salt in India in the 1990s resulted in dramatic decrease in goiter and cretinism prevalence. Vitamins began to be added to foods in Europe and North America almost a century ago. Margarine was fortified with vitamin A in Denmark (Bloch 1931) and milk with vitamin D in the United States (Park *et al*. 2000). About 98-99% of the milk supply in United States is fortified with 10 micrograms (equal to 400 International Units) of vitamin D per quart (0.946 litre). About 25-50% of additional vitamin A in the diet of the average European now comes from fortified food products. Some developing countries mainly in Central America and few in Africa have decades of experience in the application of fortification to the reduction of vitamin A deficiency (NNP 1993). From the early 1940s onwards, the fortification of cereal products with thiamine, riboflavin and niacin became common practice. Today, the fortification of flour provides the population of the United States and Canada with about a quarter of its daily iron intake. Foods for young children were fortified with iron, a practice which has substantially reduced the risk of iron deficiency anaemia in this age group. In more recent years, folic acid fortification of wheat has become widespread in the Americas, a strategy adopted by Canada and the United States and about 20 Latin American countries (Allen *et al*. 2006).

Thus, for the large and expanding populations which regularly purchase staple foods like wheat, oil, rice and milk, staple food fortification lends a cost effective and sustainable solution as it is generally socially acceptable, requires minimal change in food habits, usually costs <2% of the cost of the unfortified food and the delivery system is in place.

2.3.3 Selection of food vehicle

Selection of a suitable food vehicle and compatible fortificant are fundamental criteria for justifying intervention in the form of food fortification. In practice, selection of a food vehicle-fortificant combination is governed by various factors, both technological and regulatory. The choice of fortificant is often a compromise between reasonable cost, bioavailability from the diet, interaction with food matrix and other food components and the acceptance of any sensory changes. Safety is also an important consideration. The level of consumption that is required for fortification to be effective must be compatible with a healthy diet (www.nutritionimprovement.com, accessed on August 23, 2010).

2.3.3.1 Milk as a vehicle for vitamin A and iron fortification

Milk and milk products provide a convenient and useful vehicle for fortification with micronutrients. Milk and other dairy products are a part of the daily diet in almost all countries. Dairy products are also easily targeted for specific consumer audiences, such as females and infants, allowing for the delivery of category specific functional ingredients.

Milk in its natural form is almost unique as a balanced source of man’s dietary needs. Milk and milk products provide a convenient and useful vehicle for fortification with micronutrients and has following benefits (Nair *et al.* 2003):

- Since milk is centrally processed, hence quality control can be effectively implemented.
- Milk and milk products are widely consumed regularly in predictable amounts by people of all age groups.
- Cost is affordable by target population.
- Stability and bioavailability of the added micronutrients in milk remains high.

2.3.4 History and successful interventions

Fortification of milk with vitamins commenced in early nineties. Vitamin D fortification of milk in the United Kingdom is reported to have begun in 1923. Some dairies in USA fortify milk with vitamins C, E and calcium; in addition to vitamins A and D. Several countries have established mandatory fortification of milk since early in this century. Additionally, several other countries voluntarily add micronutrients to milk, particularly low-fat or fat-free liquid and dried milks (Raunhardt and Bowley 1996). Dried milk and flavoured milk powders are often fortified with vitamins A and D, calcium and iron. Milk-based infant formula and weaning foods are fortified with a range of vitamins, minerals and other nutrients such as polyunsaturated fatty acids. Chile demonstrated elimination of anaemia by fortifying milk powder with iron coupled with vitamin C (Torrejon *et al.* 2004). Similar programme was adopted by Argentina by fortifying milk with iron through ferrous sulphate encapsulated with phospholipids. The existing US regulation (Pasteurised Milk Ordinance) stipulates vitamin A fortification in all reduced fat milk products. Government of India too incentivised fortification of milk with vitamin A in early 1960. Prevention of Food Adulteration (PFA) Act, 1954 also stipulates that “the food claimed to be enriched with nutrients such as vitamins shall give the quantities of such added nutrients on the label.”

During 1980s, the Department of Food and Public Distribution introduced a scheme of fortification of milk with vitamin A to prevent nutritional blindness. The Government reimbursed the cost of vitamin A premix to the dairies for fortifying the toned and double toned milk. The total quantity of milk fortified with vitamin A during 1988-89, was 3.2 million litres per day at a level of 2000 IU/litre of milk (NNP 1993).

2.3.5 Technology of liquid milk fortification

The technology of milk fortification is relatively simple and no additional equipments are needed or can be practiced with minor modifications in the existing plant. All the vitamins and minerals that can be added to milk are available in dry powder form. The fat soluble vitamins are also available in an oily form. Mineral/vitamin fortification can be practiced at several stages in the production. However, liquid milk is usually fortified prior to pasteurisation or ultra-high temperature treatment. Homogenisation is especially crucial for oily preparations of vitamins. A model flow chart of liquid milk fortification (O’Brien and Robertson 1993) is presented in figure 2.1.

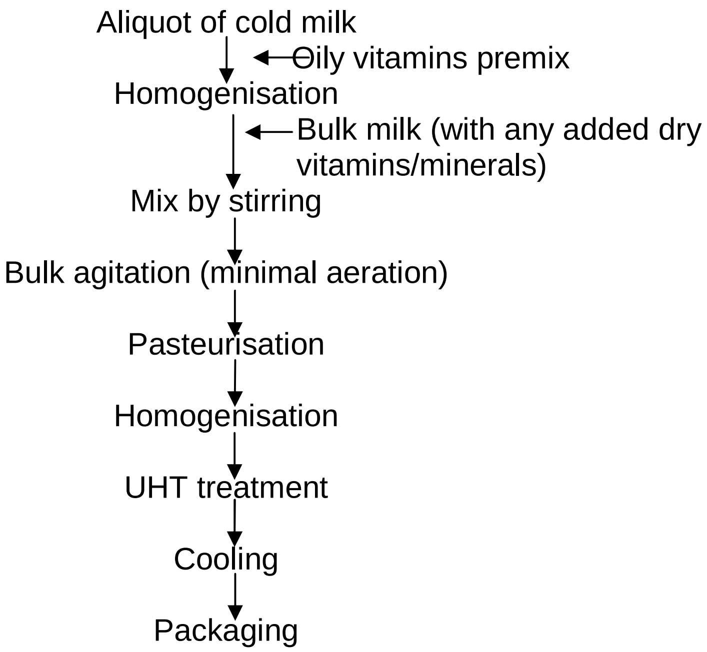

Figure 2.1: Process flow chart of liquid milk fortification

2.4 Iron fortification

Iron fortification of foods is regarded as the most cost effective method for reducing the prevalence of nutritional iron deficiency (Huma *et al*. 2007).

2.4.1 Physiological significance of iron

Iron is an essential micronutrient because it plays a vital role in many metabolic processes including oxygen transport, oxidative metabolism, cellular proliferation and growth. It is a redox metal and participates in most of the reversible redox reactions by switching between the two oxidation states, ferrous and ferric. This redox activity of iron can produce free radicals responsible for cell signaling processes and iron mediated toxicity. Iron is also an essential mineral for all known pathogens (Allen *et al.* 2006). In human beings, it is absorbed primarily in the duodenum, transported through the blood stream and extracellular fluid bound to transferrin and stored intracellularly predominantly in the form of ferritin. Iron balance is rigorously controlled by the regulation of its absorption.

2.4.2 Iron absorption

Many components of diet as well as a person's nutritional status with respect to nutrients other than iron can have a significant impact on both iron absorption and internal iron metabolism. Iron is widely distributed in human food items (Bothwell *et* *al*. 1979). Animal tissues contain iron in the form of haeme, ferritin and hemosiderin as well as iron bound to membranes and low molecular weight compounds. Iron in other animal-derived foods may also be bound to specific proteins such as lactoferrin in milk, ovotransferrin in egg white and phosvitin in egg yolk. Plant foods contain iron in the form of metalloproteins, plant ferritins, iron present in the sap and iron complexed to structural components or storage compounds predominantly as phytates (Hazell 1985). In addition, foods may contain contaminant inorganic iron salts such as ferric oxides and hydroxides (Cook 1990) or iron compounds added during processing to fortify the diet.

2.4.3 Sources

Dietary sources of iron are found in two forms: haeme iron and non-haeme iron (Chen and Oldwage 2002). Haeme sources are provided by animal tissues (meats) and are readily absorbed. Approximately 40% of iron found in meat is haeme, with the best sources being liver, seafood, fish, lean meat and poultry. Non-haeme iron is provided from plant sources and elemental components of animal tissues. It is less efficiently absorbed and the amount absorbed depends upon the body's needs. If there are low stores, more iron will be absorbed and vice-versa. Non-haeme sources that are high in iron include cooked spinach, beans, eggs, nuts, fortified breads, cereals and flours.

2.4.4 Iron requirements

Basal iron loss forms the fundamental requirement for all. Actual body iron losses amount to approximately 1 mg/day in healthy, adult men (Green *et al*. 1968; Hallberg *et al*. 1998) and are normally adequately covered by dietary iron absorption (Cook 1990; Hulten *et al*. 1995). However, regular blood losses cause menstruating women to face an additional loss of 0.5-1.5 mg iron per day (Hallberg and Rossander-Hulten 1991), which is more difficult to balance by dietary means. Likewise, children and adolescents require 0.5-1.5 mg in excess of their daily iron losses to satisfy the extra needs associated with growth. Finally, the total iron cost of pregnancy is estimated at 1040 mg, of which 200 mg are retained by the woman when blood volume decreases after delivery and 840 mg are permanently lost (Picciano 2003). The average daily requirements reach as high as 4-6 mg during the second and third trimester for foetal growth and blood volume expansion (Carpenter and Mahoney 1992). Net iron losses from the body through the skin, urine and blood are about 2 mg/day for women, 1 mg/day for men and 0.8 mg/day for children, reflecting that net iron requirements are indeed very low. Recommended dietary allowance (RDA) of iron is much higher than net iron requirement, taking into account the poor bioavailability of dietary iron (Herbert 1987). Recommended dietary allowances of iron for Indians have been shown in table 2.1.

Table 2.1: Recommended dietary allowances of iron for Indians

Group	**Recommended dietary allowances**			
	Body weight (kg)	**Requirement (µg/kg/d)**	**Absorption assumed (%)**	**RDA (mg/d)**
Adult Man	60	14	5	17
Woman (NPNL)	55	30	8	21
Pregnant woman	55[a]	51	8	35
Lactating woman (0-6 m)	55	23	8	25
Infants 0-6 m	5.4	46	---	---
6-12 m	8.4	87	15	5
Children 1-3 y	12.9	35	5	9
4-6 y	18.0	35	5	13
7-9 y	25.1	31	5	16
Adolescents Boy 10-12 y	34.3	31	5	21
Girl 10-12 y	35.0	38	5	27
Boy 13-15 y	47.6	34	5	32
Girl 13-15 y	46.6	29	5	27
Boy 16-17 y	55.4	25	5	28
Girl 16-17 y	52.1	25	5	26
Source: ICMR 2009				

[a] Pre-pregnancy weight

NPNL: non-pregnant non-lactating

2.4.5 Iron deficiency

The main risk factors for iron deficiency in body are low intake of haeme iron and vitamin C, poor absorption of iron from foods high in phytates, periods of life when iron requirements are especially high (i.e. growth and pregnancy); and heavy blood losses in menstruation or parasite infections. Iron deficiency induces anaemia which affects more than 30% of the world's population, especially infants, women and teenagers. Around 60% women of reproductive age and 40-50% teenagers are affected by iron deficiency. Fortification of milk with iron and vitamin C (ascorbic acid) in Chile produced a rapid reduction in the prevalence of iron deficiency in infants and young children (Stekel *et al.*1988; Hertrampf 2002).

2.4.6 Safety issues

Higher levels of iron intake and elevated body stores are potential risk factors for both coronary heart disease (CHD) and cancer. Inflammatory response is a risk factor for CHD and also increases serum ferritin, which might explain why an association between the risk of CHD and increased serum ferritin is sometimes observed (Danesh and Appleby 1999). It has been hypothesised that the presence of unabsorbed fortificant iron in the body, much of which reaches the colon, leads to free radical generation that damages the colon mucosa (Lund *et al.* 1999). However, there is no evidence that the free radicals survive long enough to cause tissue damage.

2.4.7 Choice of iron fortificant

Technically, iron is the most challenging micronutrient to be added to foods. Iron compounds that have the best bioavailability tend to be those which interact most strongly with food constituents to produce undesirable organoleptic changes. The ideal product for food fortification is one that supplies highly bioavailable iron, does not diminish the nutritional value of the food vehicle through nutrient oxidation, does not alter its sensory properties, can be used to fortify solid and liquid foods, is resistant to food technology processes and is low in cost so that it can be accessible to the whole population (Boccio *et al*. 1996).

The selection of iron compound for fortification is important in order to avoid interactions of iron with food vehicle or the total meal because a minor change in organoleptic characteristics of the food will result in consumer rejection. When the iron compound is added, it is necessary to evaluate possible changes in food colour, taste or appearance with time and storage under adverse temperature and humidity conditions. Solubility, chemical reactivity, bioavailability and cost are other important issues when selecting an iron compound. For instance, ferrous sulphate is a highly bioavailable and relatively inexpensive compound, however, because of its high reactivity, it produces undesirable changes in some fortified foods. On the other hand, elemental iron (reduced, electrolytic or carbonyl) is also inexpensive, however, it has been reported to have a low bioavailability depending on particle size and the food vehicle to be fortified (Hallberg 1981; Cook 1983; Hallberg *et al*. 1986b).

2.4.7.1 Types of iron fortificants

A wide variety of iron compounds are currently being used as food fortificants (table 2.2). These can be broadly divided into four categories:

2.4.7.1.1 Water soluble compounds

Being highly soluble in gastric juices, the water soluble iron compounds have the highest relative bioavailability amongst all the iron fortificants. However, these compounds are also most likely to have adverse effects on the organoleptic properties of foods, in particular, on the colour and flavour. During prolonged storage, the presence of fortificant iron in certain foods can cause rancidity and subsequent off-flavours. Moreover, in the case of multiple fortification, free iron, produced from the degradation of iron compounds present in the food, can oxidise some of the vitamins supplied in the same fortificant mixture. The water-soluble forms of iron are especially suited to fortify cereal flours. Water soluble iron compounds are also useful for dry foods, such as pasta and milk powder, as well as dried milk-based infant formulas.

Among the various iron fortificants, ferrous sulphate is a water soluble compound that has the highest relative bioavailability among conventional iron compounds (Hurrell and Cook 1990), however, it is relatively unstable and may reduce vehicle-food quality and shelf life due to potential oxidised off-flavours, colour changes and metallic flavours (Jackson and Lee 1991).

2.4.7.1.2 Iron compounds that are poorly soluble in water but soluble in dilute acids

This type of iron fortificants are also reasonably well absorbed from food, as they are soluble in the gastric acids produced in the stomach of normal healthy adults and adolescents. In most people, iron absorption from these compounds is likely to be similar to that from water soluble iron compounds. Poorly water soluble iron compounds, such as ferrous fumarate, have the advantage of causing fewer sensory problems in foods than the water soluble compounds, and are generally next in line for consideration, especially if more water soluble forms cause unacceptable organoleptic changes in the chosen food vehicle. Ferrous fumarate and ferric saccharate are the most commonly used iron compounds in this group and are as bioavailable as ferrous sulfate. The former is frequently used to fortify infant cereals and the latter to fortifiy chocolate drink powders (Allen *et al*. 2006)

2.4.7.1.3 Iron compounds that are insoluble in water and poorly soluble in dilute acids

Relative to ferrous sulfate, the absorption of iron from water insoluble compounds ranges from approximately 20 to 75%, however, these have far less effects on the sensory properties of food. However, they are generally regarded as the last resort option, especially in settings where the diet of the target population is high in iron absorption inhibitors. Within this category of iron fortificants, the ferric phosphate compounds i.e. ferric orthophosphate and ferric pyrophosphate are used to fortify rice and some infant cereals and chocolate containing foods. The relative bioavailability of ferric pyrophosphate is reported to be 21–74% and that of ferric orthophosphate 25-32% (Theuer *et al*. 1973; Hurrell *et al*. 1991).

Elemental iron is half bioavailable and more stable as compared to ferrous sulphate (Hallberg *et al*. 1986b). If elemental iron is used as the fortificant, higher doses (two or three times more than those required for ferrous sulphate) may be needed to compensate for the lower bioavailability. This makes the cost of fortification with elemental iron higher as compared with ferrous sulphate. Elemental iron in wheat flour fortified at a higher rate also had an adverse effect on the chapatti quality, however, there is contradiction among different studies, as the preparation and baking method are different (Hinnai *et al*. 2000; Rehman *et al*. 2003).

2.4.7.1.4 Encapsulated or protected forms

Novel microencapsulation technologies render iron fortificants that are more resistant to interaction with other components in the food vehicles, thus minimising organoleptic changes, increasing shelf life and maximising consumer acceptance. Most of the iron compounds can be microencapsulated, however, the most available products are the microencapsulated form of both ferrous sulphate and ferrous fumurate (Boccio *et al*. 1997). The coatings are usually a mixture of phospholipids, polysaccharides, protein or partially hydrogenated oil. Microencapsulation has little influence on relative bioavailability and the main advantages are fewer organoleptic changes and a prolonged shelf life of fortified foods. This is a result of the bilayer coating, protecting against the interaction between iron and absorption factors in the fortified foods.

Encapsulation increases cost 3- to 5-folds, which when expressed in terms of iron amounts, is equivalent to a 10-folds increase in cost relative to the use of dried ferrous sulfate. The main purpose of encapsulation is to separate iron from the other food components, thereby mitigating sensory changes (Hurrell 1999, 2002; Swain *et al.* 2003). When developing encapsulated iron fortificants, it is important to select a coating material which provides an adequate balance between stability and bioavailability. Iron compounds are usually encapsulated with hydrogenated vegetable oils, however, mono- and diglycerides, maltodextrins and ethyl cellulose, have also been used (Allen *et al*. 2006).

Iron compounds listed as generally recognised as safe (GRAS) by United States Food and Drug Administration (USFDA) include elemental iron, ferrous ascorbate, ferrous carbonate, ferrous citrate, ferrous fumarate, ferrous gluconate, ferrous lactate, ferrous sulphate, ferric ammonium citrate, ferric chloride, ferric citrate, ferric pyrophosphate and ferric sulphate (Whittaker 1998).

2.4.8 Novel iron fortificants

In recent years, considerable efforts have been devoted to the development and testing of alternative iron fortificants, in particular, fortificants that provide better protection against iron absorption inhibitors than those currently available. Among those at an experimental stage are monosodium ferric EDTA (NaFeEDTA), ferrous bisglycinate and various encapsulated and micronised iron compounds. In high-phytate foods, the absorption of iron from NaFeEDTA is 2–3 times greater than that from either ferrous sulfate or ferrous fumarate. In foods with low phytate content, however, iron absorption is similar (INACG 1993 and Hurrell *et al*. 2000). The Joint FAO/WHO Expert Committee on Food Additives has approved the use of NaFeEDTA at 0.2 mg iron/kg body weight per day (FAO/WHO 1999).

Table 2.2: Key characteristics of iron compounds used for food fortification purpose: solubility, bioavailability and cost

Compound	Iron content (%)	Relative bioavailability[a]	Relative cost[b] (per mg iron)
1. **Water soluble**			
Ferrous sulphate. 7 H_2O	20	100	1.0
Ferrous sulphate, dried	33	100	1.0
Ferrous gluconate	12	89	6.7
Ferrous lactate	19	67	7.5
Ferrous bisglycinate	20	>100[c]	17.6
Ferric ammonium citrate	17	51	4.4
Monosodium ferric EDTA	13	>100[c]	16.7
2. Poorly water soluble, soluble in dilute acids			
Ferrous fumarate	33	100	2.2
Ferrous succinate	33	92	9.7
Ferric saccharate	10	74	8.1
3. Water insoluble, poorly soluble in dilute acids			
Ferric orthophosphate	29	25-32	4.0
Ferric pyrophosphate	25	21-74	4.7
Elemental iron			
H-reduced	96	13-148[d]	0.5
Atomised	96	(24)	0.4
CO-reduced	97	(12-32)	<1.0
Electrolytic	97	75	0.8
Carbonyl	99	5-20	2.2
4. Encapsulated forms			
Ferrous sulphate	16	100	10.8
Ferrous fumarate	16	100	17.4

EDTA: ethylenediaminetetraacetate; H-reduced: hydrogen reduced; CO-reduced: carbon monoxide reduced.

a. Relative to hydrated ferrous sulphate ($FeSO_4$. $7H_2O$), in adult humans. Values in parenthesis are derived from studies in rats.

b. Relative to dried ferrous sulphate per mg of iron, the cost of hydrated and dry ferrous sulphate in similar.

c. Absorption is two-three times better than from ferrous sulphate if the phytate content of food vehicle is high.

d. The high value refers to a very small particle size which has only been used in experimental studies.

Sources: Hurrell 1999, 2002; Swain *et al.* 2003

2.4.9 Methods used to increase the amount of iron absorbed from fortificants

The bioavailability of iron from fortificants is dependent not only on the solubility of the fortificant, but also on the composition of the diet, in particular, on the proportion of inhibitors of iron absorption in the diet, notably iron-binding phytates and certain phenolic compounds. The addition of ascorbic acid (vitamin C) or sodium ethylenediaminetetraacetic acid (Na_2EDTA) and the removal of phytates, all of which reduce the effect of the inhibitors, can be effective ways of increasing the total amount of iron absorbed from iron fortified foods.

2.4.10 Food vehicles for iron fortification

2.4.10.1 Cereal products

Cereal flours are currently the most frequently used vehicles for iron fortification that reach the entire population. The amount of iron added is usually relatively low because it is added only to restore the iron level in milled flour to that of whole grain. With true fortification, a higher amount than is usually present would be added. The contribution of fortified iron to iron intake is highest in the United States, where it accounts for 20-25% of total iron intake. The contribution of fortified iron to iron intake in the United Kingdom is much lower i.e. around 6% (Hurrell 1997).

Wheat flour enrichment is mandatory in many countries. Recently, it has been proposed that wheat flour in Central America to be fortified with 60 mg/kg of flour with reduced electrolytic iron of particle size less than 40 μm (Bedolla and Rooney 1984).

Technology also exists for fortifying whole grains such as rice. This can be done by coating, infusing or by using extruded grain analogues. The fortified grains are then mixed 1:100 or 1:200 with the normal grains. Hunnell *et al*. (1985) described a sophisticated method of preparing fortified rice grains by first infusing B vitamins and then adding iron, calcium and vitamin E in separate layers of coating material. There are two major disadvantages in using cereal products as vehicles for iron fortification. First, they contain high levels of phytic acid, a potential inhibitor of iron absorption upto 1% in whole grains and about 100 mg/100 g in high-extraction flours (Davidsson 2003). Second, these are extremely sensitive to fat oxidation during storage when highly bioavailable iron compounds such as ferrous sulphate are added (Hurrell *et al*. 1989).

For organoleptic reasons, cereal flours such as wheat and maize are usually fortified with poorly absorbed elemental iron powders and rice with ferric orthophosphate or ferric pyrophosphate (Hunnell *et al*. 1985). Only bread and wheat flour stored for less than 3 months and pasta products, because of their low moisture content, can be fortified with highly bioavailable ferrous sulphate (Barret and Ranum 1985). However, even in these foods, iron absorption will be inhibited by the presence of phytic acid, unless an absorption enhancer is present (Hurrell 1997).

2.4.10.2 Salt

Iodine fortified salt has successfully eradicated iodine deficiency in many countries (Nadiger *et al*. 1980), therefore, salt would also seem a highly suitable vehicle for iron fortification. However, iron fortification of salt poses many technical problems, and for developing countries, an efficient production and distribution system must also exist. Almost all of the developmental work for the fortification of salt with iron has been conducted in India. Colour changes during storage have been the main problem, because salt in India is relatively crude and contains upto 4% moisture. All soluble iron compounds and vitamin C caused unacceptable colour changes (Hurrell 1997). Fortification was possible only with insoluble iron compounds and ferric orthophosphate was recommended at a level of 1 mg per g salt so as to provide about 15 mg extra iron per day. When $NaHSO_4$ (sodium hydrogen sulphate) was added as an absorption promoter, absorption was reported to be 80% to that of ferrous sulphate.

A small scale fortification trial in which the fortified salt was included in a school feeding program demonstrated an improvement in iron status in India (Andersson *et al*. 2008). Salt that contains fewer impurities would undoubtedly be easier to fortify, however, the extra cost to the consumer is always a major consideration in developing countries. In addition, there is always the possibility that the iron fortified salt will cause unacceptable colour reactions if added to vegetables in a meal. This was one of the explanations offered for the failure of a fortification program in the Seychelles and Mauritius in the early 1960s (Foy 1976). The other reasons were the relatively poor bioavailability of the ferric pyrophosphate used and the fact that it separated from the salt and sank to bottom of salt barrels.

Overcoming all the difficulties, the scientists at National Institution of Nutrition (NIN), Hyderabad, India, have developed a technology for double fortification of salt with iodine and iron (www.fnbnew.com, accessed on August 19, 2011)

2.4.10.3 Sugar

Sugar is an alternative vehicle for iron fortification in the regions of the world where it is produced, such as the Carribean and Central America, however, in other developing countries, refined sugar consumption is more common in middle and upper socio-economic segments of the population (Lynch *et al*. 1993). Iron from fortified sugar would be expected to be well absorbed if consumed with citrus drinks but poorly absorbed from coffee and tea owing to phenolic compounds or, if added to cereal products, owing to phytates. As with salt, the main technical problem is to select a bioavailable iron compound that does not cause unwanted colour changes in less pure sugar products.

In Guatemala, this was overcome by adding monosodium ferric EDTA (NaFeEDTA). Commercially, white cane sugar would appear easier to fortifiy. Disler *et al.* (1975a) reported the successful addition of different ferric and ferrous compounds (100-200 mg iron/kg) together with vitamin C to white cane sugar. There were, however, unacceptable colour reactions when added to coffee and tea or to certain maize products. The use of sugar fortified with iron tris-glycinate chelate in the prevention of iron deficiency anaemia in preschool children in Brazil was reported to be effective (Paula and Fisberg 2001).

2.4.10.4 Condiments

Condiments that are traditionally used in developing countries, such as monosodium glutamate, fish sauce, curry powder and bouillon cubes, could be useful fortification vehicles (Lynch *et al*. 1993). Monosodium glutamate (MSG) is widely used as a flavour enhancer in Asia and has been successfully fortified with ferric orthophosphate and ferrous sulphate encapsulated in zinc stearate. Pilot fortification trials with fish sauce or curry powder, both fortified with monosodium ferric EDTA, resulted in significant improvement in iron status in the population consuming the fortified products (Ballot *et al*. 1989; Garby and Areekul 1974). The success of fortified condiments presumably depends on the absence of adverse colour reactions and on the addition of an absorption enhancer, such as EDTA.

2.4.10.5 Coffee

Coffee is widely consumed in some populations, by most adults as well as some children, and it is technically and economically feasible to fortify coffee with iron. Johnson and Evans (1977) reported the use of ferrous fumarate in roasted and ground coffee, in which one cup (200 ml) provided 1 mg added iron. The addition of iron to soluble coffee is also relatively easy as reported by Klug *et al*. (1973), who found that addition of a range of soluble ferrous and ferric compounds was possible. Flavour and colour changes, however, are potential problems, and coffee, like tea and cocoa, contains phenolic compounds that strongly inhibit iron absorption.

2.4.11 Applications of iron fortification in the dairy industry

2.4.11.1 Cheddar cheese

Cheddar cheese was manufactured from iron supplemented milk (Zhang and Mahoney 1988, 1989b, 1990, 1991). Different sources and levels of iron were used. Iron recoveries in the cheeses were 71-81% for ferric chloride, 52-53% for ferric citrate, 55-75% for iron-casein complex and 70-75% for ferripolyphosphate–whey protein complex. TBA numbers increased slightly in iron fortified cheeses but were within the range reported by others for unfortified cheeses. Aging cheeses up to three months did not change the TBA assay or oxidised off-flavour and cheese flavour scores. Cheddar cheese fortification (40-50 mg/kg) with ferripolyphosphate-whey protein complex, ferric-casein complex and ferric chloride was possible without affecting its quality.

2.4.11.2 Mozzarella cheese

Mozzarella cheese containing 25 and 50 mg iron/kg cheese was manufactured from milk fortified with casein- or whey protein-chelated iron or ferric chloride (Rice and McMahon 1998). Addition of 25 mg iron/kg of cheese had no effect on the physical properties of cheese. Apparent viscosity of cheese fortified with 50 mg of iron/kg of cheese tended to be slightly higher than the control cheese, although this difference was not significant at all storage times. No increase in chemical oxidation was observed between the control and iron fortified cheeses.

Slight increase in metallic and oxidised flavours was observed in the iron fortified cheese. Sensory scores for iron fortified cheese made using casein-chelated iron or whey protein-chelated iron were not significantly different from those of cheese made using ferric chloride (Rice and McMahon 1998).

2.4.11.3 White soft cheese

A white soft cheese was produced from pre-cheese retentate fortified with electrolytic iron, ferric chloride and ferrous sulphate at a level of 80 mg iron/kg (El-Samragy *et al*. 1995).

2.4.11.4 Baker's and cottage cheese

Skim milk fortified with ferric ammonium citrate was used for the manufacture of Baker's and cottage cheese (Sadler *et al*. 1973). Approximately 14% of the iron was retained in Baker's cheese and 58% in the cottage cheese. Added iron had no effect on starter activity or on the initial flavour of the product. During storage for 2 months, no accelerated development of off-flavours was observed in the iron fortified cheese.

2.4.11.5 Infant foods

Infant formula is an excellent vehicle for iron fortification as it is consumed by the non-breastfed infants in large quantities. Further, the commercially available infant formulas, which are either milk based or soy based products, can be fortified with readily bioavailable ferrous sulphate without causing discolouration or fat oxidation. Ziegler and Fomon (1996) reported that infant formulas always contain ascorbic acid (minimum levels approximately 50 mg ascorbic acid per litre formula) which would further increase the amount of available iron. Presently, formulas (for healthy term infants) in the United States are commonly fortified with 12 mg iron/litre, in agreement with the recommendation of the American Academy of Pediatric's Committee on Nutrition (1999). Dried whole milk powders and dried or ready-to-feed milk-based infant formulas can be successfully fortified with ferrous sulfate (together with ascorbic acid to enhance absorption). In Chile, ascorbic acid (700 mg/kg) and iron (100 mg as ferrous sulfate/kg) are routinely added to dried milk powders consumed by infants.

In Europe, infant formulas should contain between 3 and 10 mg iron/litre, although formulas with no added iron are permitted providing a statement is made to the effect that, when the product is given to infants over the age of 4 months, their total iron requirements must be met from other additional sources (Moy 2000). Ziegler and Fomon (1996) reported that in the United States, “unfortified” or low-iron infant formulas contain small amount of added iron (1.5-4.0 mg/L). It is however, discussed whether the iron fortification levels, especially those in the United States, are too high (Dallman 1989; Moy 2000). This is based on the findings showing that iron absorption increases with decreasing iron content (Sarrinen *et al*. 1977; Fomon *et al*. 1997). Further, long term evaluations (0-9, 1.5-6 or 6-12 months of age) have shown that infant formulas with low iron content can be just as effective in preventing iron deficiency as those with high iron content (Lonnerdal and Hernell 1994; Walter *et al*. 1998).

Infant cereals are commonly fortified with iron. Hurrell *et al*. (1989) and Theuer (2002) have suggested that less soluble iron compounds are used to avoid organoleptic problems since ferrous sulphate provokes off-colours as well as fat oxidation. These include elemental iron powders in United States and ferric pyrophosphate and ferric orthophosphate in Europe (Hurrell 1989). It is difficult to fortify infant cereals with readily available iron compounds, since these have the further disadvantage of containing phytic acid. However, the inhibitory effect of phytic acid can be overcome by the addition of ascorbic acid (Davidsson *et al*. 1997).

2.4.11.6 Yoghurt

Yoghurts (non-fat and low fat) were manufactured and fortified with 10, 20 and 40 mg of iron/kg of yoghurt (Hekmat and McMahon 1997). Counts of *Lactobacillus delbruckii* ssp. *bulgaricus* and S*treptococcus thermophilus* after 1 day of storage in iron fortified skim yoghurts were 7.0×10^8 cfu/ml which were similar to numbers in unfortified yoghurts. No increase in chemical oxidation was detected. Iron fortified yoghurts had slightly higher oxidised flavour scores than the control yoghurt. There was no increase in metallic, bitter or other off-flavours. The consumer panel did not detect any differences in the appearance, mouthfeel, flavour or overall quality between fortified and unfortified flavoured yoghurts.

2.4.11.7 Iron fortified chocolate milk

Chocolate milks, fortified with iron compounds were evaluated for changes in colour and flavour (Douglas *et al*. 1981). Sodium ferric pyrophosphate, ferripolyphosphate and ferripolyphosphate–whey protein complex produced little or no off-colour change in the products initially and after 2 weeks of storage. All other added compounds resulted in initial and persistent off-colours. Flavour evaluation showed that ferric compounds produced little or no off-flavours in chocolate milks initially or after holding at 4ºC for 7 and 14 days. Ferrous compounds produced off-flavours initially, however, flavour scores improved after milks were held at 4ºC for 14 days (Douglas *et al*. 1981). Iron fortification of milk has always been difficult because of production of undesirable colour and flavour changes due to iron stimulated autoxidation of milk fat. Kinder *et al*. (1942) proposed that cocoa and chocolate contain natural antioxidants which would prevent development of oxidative rancidity in chocolate milk.

2.4.11.8 Iron fortification of milk

2.4.11.8.1 Natural concentration and distribution of iron in milk

Milk is relatively poor in iron (0.2 mg/kg) (Flynn 1992). This content varies from cow to cow and depends on the stage of lactation but not on the feed. 14% of the iron occurs in milk fat where it is associated with the fat globule membrane (Fransson and Lonnerdal 1983). About 24% of the iron is bound to casein while 29% is bound to whey proteins and 32% is associated with a low molecular weight fraction. In skim milk, 50-65% of the iron is bound to caseins, 18-33% is in the whey proteins fraction and 15–33% is in the non-protein fraction (Brule´ and Fauquant 1982). This fraction and the low molecular weight fraction correspond to small molecules, such as citric acid, orotic acid and Pi (inorganic phosphate). Among the whey proteins, lactoferrin can bind two Fe^{3+}, concomitantly with two carbonate ions. However, this transferrin is present at low concentration in bovine milk (0.01–0.1 mg/ml).

2.4.11.8.2 Iron distribution in iron supplemented milk

King *et al*. (1959) used Fe^{59} to determine the distribution of iron in whole milk and showed that all of the added iron is associated with the skim milk. When iron is added as ferrous chloride, ferric chloride or ferripolyphosphate to skim milk, it is bound to the colloidal phase at about 80-90% (King *et al*. 1959; Bash *et al*. 1974; Demott and Dincer 1976; Hegenauer *et al*. 1979d; Gaucheron *et al*. 1997a; Hekmat and McMahon 1998). The oxidative state of iron does not play an important role in its distribution in the milk fractions (Hegenauer *et al.* 1979d). However, the distribution of iron depends on the nature of the iron added. The use of compounds that donate their iron easily (chloride, sulfate and nitriloacetate) leads to casein modifications. On the other hand, the chelators or proteins that bind iron strongly (EDTA, caseins and whey proteins) do not modify the colloidal phase because iron is not exchangeable (King *et al*. 1959).

2.4.11.8.3 Iron fortified milk and milk products

Ferrous sulfate and many other soluble iron compounds can not be used to fortify liquid whole milk and other dairy products since they cause rancidity and off-flavours. Iron fortificants are best added after the milk has been homogenised and the fat internalised in micelles, so as to help in protecting the fat against oxidation. Ferrous bisglycinate is widely used to fortify whole milk and dairy products in Brazil and Italy. Micronised ferric pyrophosphate is added to dairy products in Japan. Ferric ammonium citrate, ferrous bisglycinate and micronised ferric pyrophosphate have been suggested for fluid milk fortification and ferrous sulphate plus ascorbic acid for dry milk (Allen *et al*. 2006).

Wang and King (1973) have reported that ferrous sulphate, ferric ammonium sulphate and citrate caused TBA values of 0.092, 0.046 and 0.023 at concentrations of iron 10 ppm only, thereby proving that ferric ammonium citrate was the best compound to be used for fortification. Kurtz *et al.* (1973) reported that ferric ammonium citrate and ferric chloride @20 ppm iron could be used for fortification of milk without causing adverse flavour effects. Olivares *et al*. (1997) suggested ferrous bisglycinate for fortification of fluid whole milk and other high fat vehicles. The good bioavailability of iron bisglycinate makes this compound a suitable alternative to be considered for iron fortification programme.

Ravikiran *et al.* (1977) showed that no appreciable flavour was detected after fortification of milk with ferric lactose at 4ºC upto 24 hrs. The combination of ascorbic acid and iron in the doses studied (50 mg ferric lactose + 50 mg ascorbic acid per 100 ml of milk) could provide an effective antioxidant system without much loss of ascorbic acid. Ferric lactose alone caused oxidation, resulting in a higher TBA value as compared to that with control (unfortified milk). Ascorbic acid not only protected the milk from fat oxidation, but it also completely suppressed oxidation of milk fat caused by ferric lactose (50 mg/100 ml). No appreciable detrimental effect could be observed on pH, flavour (organoleptic) and rancidity (lipolytic) or other physico-chemical characteristics.

Baldwin *et al.* (1982) reported that fortification of milk with reduced iron, complexed with citric acid and phosphoric acids, lowered the intensity of cooked flavour and exerted little influence on oxidised flavour in milk pasteurised at 80ºC for 25 seconds. They also concluded that the likelihood of oxidised off-flavours would be greater if the iron sources were added after pasteurisation at 72ºC for 15 seconds.

2.4.11.8.4 Microencapsulated iron for milk fortification

Direct addition of iron to milk might have detrimental effects on the quality and acceptability due to development of oxidised off-flavour, colour changes and metallic flavours (Wang and King 1973). Microencapsulation has been used for many years to protect sensitive food components, preserve desirable flavours and aroma, inhibit nutritional loss and mask undesirable flavours (Dziezak 1988).

Boccio *et al*. (1996) used a compound called SFE-171 (commercial name Biofer™), a ferrous sulfate microencapsulated with lecithin and claimed that this product had the same bioavailability as ferrous sulfate, however, had the advantage of being coated with a phospholipid membrane. This kept the iron from coming in contact with the food vehicle, prevented the undesirable interactions that happened when conventional ferrous sulfate is used.

Kwak *et al*. (2003) developed a microencapsulated iron that could be used to fortify milk and determined the sensory properties of milk fortified with microencapsulated iron. Wall material was polyglycerol monostearate (PGMS) and selected core material was ferric ammonium sulfate. The highest efficiency of microencapsulation was 75% with 5:1:30 ratio (w/w/v) as coating to core materials to distilled water. Iron release was 12% when stored at 4°C for 3 days. TBA value was lowest when 100 ppm of encapsulated iron was added to milk and was significantly lower in encapsulated groups compared with that in unencapsulated groups. In a sensory analysis, most aspects except for metallic taste and colour were not significantly different between control and encapsulated iron fortified milk at 3 days of storage. However, between encapsulated and unencapsulated groups, astringency, metallic, colour and overall scores were significantly different. Xia and Xu (2005) prepared iron containing liposomes (15 ppm iron as $FeSO_4$) using egg phosphatidylcholine, cholesterol and Tween 80 by reverse-phase evaporation method. The encapsulation efficiency (EE) was 67% and milk fortified with ferrous sulfate liposomes was stable to heat sterilisation (100ºC/30 minutes) and storage at 4ºC for one week.

2.4.12 Stability

Minerals are more resistant to manufacturing processes than vitamins. However, they do undergo changes when exposed to heat, air or light. Minerals such as copper, iron and zinc are also affected by moisture and may react with other food components such as proteins and carbohydrates (SUSTAIN 2001).

Ferrous sulphate, the most bioavailable iron compound is subjected to oxidation during storage of fortified products at higher temperature and moisture levels. During oxidation, the ferric form is converted into the ferrous form (Rehman *et al*. 2006). Ferric complexes can cause the food product to turn brown and result in formation of brown precipitates. These ferric complexes are insoluble and therefore, poorly absorbed in the intestinal lumen (Martynez-Monzo and Fito 2002). The stability can be enhanced by adding EDTA to ferrous sulphate (MacPhail *et al*. 1994; Lotfi *et al*. 1996).

2.4.13 Methods for analysis of iron

Different titrimetric and spectrometric methods are available for analysis of iron (table 2.3).

Table 2.3: Analytical methods used for iron estimation

Matrix	Method	Specification	Reference (s)
Milk	Complexometric titrimetric method	Direct titration with Na_2EDTA by constant potential amperometry (electrotitrimetry), pH 2	Rao *et al*. (1991)
Water	Redox titrimetric method	Oxidation with $KMnO_4$; titration with dilute $TiCl_3$ (AOAC 936.02)	AOAC (2002)
Infant formula dialysate	Colourimetric method	Bathophenanthroline/hydroxyl-amine hydrochloride/535 nm	Luten *et al.* (1996)
Sweets and chewing gum	ET-AAS (Electrothermal atomic absorption spectrophotometry)	Milk: dry ashing (450ºC/1 hr); slurries: (ethanol-H_2O_2-ammonium dihydrogen phosphate)	Vinas *et al.* (1994)
Flour, milk	FAAS (Flame mode atomic absorption spectrophotometry)	Dry ashing at 600ºC	Ake *et al*. (1999)
Infant formula	ICP-AES (Inductively coupled plasma atomic emission spectrophotometry)	Aqueous solution, 10% of mixed tertiary amines containing EDTA	Hua *et al.* (2000)
Skimmed milk, yoghurt	FAAS (Flame mode atomic absorption spectrophotometry)	Dry ashing at 460ºC	Rojas *et al.* (2000); Sanchez-Segarra *et al.* (2000)

2.4.14 Biophysicochemical modifications in iron supplemented milk

2.4.14.1 Decrease in pH

Addition of ferrous chloride ($FeCl_2$) and ferric chloride ($FeCl_3$) to skim milk led to a decrease in pH (Gaucheron *et al*. 1997a). This decrease is related to the acidities of iron solutions and to exchanges between iron ions and micellar bound H^+. The characterisation of iron-phosphopeptide β-casein (β-CN) (1–25) complexes by electrospray ionisation mass spectrometry shows the release of three protons for one bound iron atom (Gaucheron *et al*. 1995). A similar pH decrease is observed after iron addition to phosvitin, traducing a proton displacement by iron from the phosphate groups that are partially protonated (Taborsky 1991).

2.4.14.2 Casein modifications

2.4.14.2.1 Iron binding to phosphopeptide in model solutions

The clusters of phosphoseryl residues (Pser) present in the caseins have the properties to bind Ca, Mg, Zn, Mn and Fe (Baumy and Brule´ 1988; Bouhallab *et al*. 1991; Meisel and Schlimme 1993; Gaucheron *et al*. 1996, 1997b). Analysis of a β-CN tryptic digest in the presence of ferrous chloride ($FeCl_2$) by mass spectrometry revealed that only phosphopeptide (1-25), which has 4 Pser, is able to bind iron (Gaucheron *et al*. 1995). These complexes between iron and phosphopeptides can be produced in industrial quantity and can be used for the development of infant and dietetic formulas (Meisel and Schlimme 1993).

2.4.14.2.2 Iron binding to caseins: changes in their charge and structure

The iron binding to caseins depends on their nature (a_{s1}-, a_{s2}-, β- and K-CN) and the nature of the iron compounds. When the compound donates its iron easily, Pser of caseins are the main binding sites (Hegenauer *et al*. 1979b). The binding of iron to caseins induces a neutralisation of their negative charges and an enhancement of hydrophobic interactions leading to their aggregations. The initial charge (2+ or 3+) of iron is important since the caseins precipitate at concentrations of 2 or 4 mM in the presence of ferrous chloride ($FeCl_2$) or ferric chloride ($FeCl_3$), respectively (Gaucheron *et al*. 1996, 1997b). The binding of iron to caseins induces a change in their structures (Reddy and Mahoney 1991).

2.4.14.2.3 Iron binding to casein molecules in milk

Iron in iron supplemented skim milk is mainly bound to a_{s1}-CN which has 7–9 Pser (Demott and Dincer 1976; Gaucheron *et al*. 1997a; Hekmat and McMahon 1998). Iron binding to β-CN, which has 5 Pser and to $α_{s2}$-CN (10-13 Pser) is also possible but in lower proportion. In milk, the iron binding to caseins may be different than in model solutions because in milk, the Pser interact with calcium phosphate salts. Moreover, the physico-chemical environment (pH, ionic strength and association of casein molecules) is different from those described in model systems (Gaucheron 2000).

2.4.14.2.4 Decrease in casein micelle hydration

After iron supplementation of milk, the binding of ferrous ion (Fe^{2+}) or ferric iron (Fe^{3+}) to caseins induces a decrease in casein micelle hydration. This depends on the nature of iron salt and is more pronounced in the presence of ferric chloride ($FeCl_3$) than ferrous chloride ($FeCl_2$) (Gaucheron *et al*. 1997a).

2.4.14.3 Whey protein modifications

The whey protein fraction is slightly modified by iron supplementation of milk (5–10% of total iron added) (Demott and Dincer 1976). However, the nature of the whey proteins modified by iron is not determined precisely. Some model studies on the interaction between iron and purified α-lactalbumin (α-la) and β-lactoglobulin (β-lg) indicate that these proteins bind iron. α-la and β-lg can bind 6 and 3.5 Fe^{2+} (ferrous ions), respectively (Baumy and Brule´1988). Their binding abilities decrease with the lowering of pH. The binding sites for Fe^{2+} (ferrous ion) consist probably of carboxylic groups of Glu and Asp, the dissociation of which is influenced by pH (Gaucheron 2000).

2.4.14.4 Modifications of milk salt balance

The modifications of salt balance after iron supplementation have not been extensively studied. Modifications of the mineral equilibrium are generally consequences of the iron binding to caseins. When iron reacts with caseins, the effect on salt balance depends on the nature of the iron compounds. Thus, in the presence of iron; calcium, phosphate (Pi) and citrate in the milk aqueous phase decrease linearly with iron concentration although in the presence of Fe^{3+} (ferric ion), only phosphate (Pi) and citrate decrease (Gaucheron *et al*. 1997a).

2.4.14.5 Change of iron oxidation state

The formation of iron-casein complexes induces the oxidation of iron from ferrous to ferric state (Manson and Cannon 1978). Dissolved oxygen serves as an oxidant which is demonstrated by the absence of oxidation when the reaction is carried out anaerobically. The oxidation rate is proportional to the casein concentration (Emery 1992).

2.4.14.6 Change in flavour and lipid oxidation

Iron is known to catalyse lipid oxidation resulting in rancidity with development of an unpleasant odour and flavour. Fortification with ferric chloride ($FeCl_3$), ferrous sulphate ($FeSO_4$) or ferric/ferrous ammonium sulfate cause oxidised off-flavour and high TBA (2-thiobarbituric) number (Demott 1971; Edmonson *et al*. 1971; Kurtz *et al*. 1973; Wang and King 1973; Hegenauer *et al*. 1979c; Douglas *et al*. 1981). Ferrous salts produce the greatest oxidation and ferric chelates the least; polynuclear complexes are intermediate (Hegenauer *et al*. 1979c). Demott (1971) found that ferric phosphate, ferric pyrophosphate or ferric ammonium citrate when added to raw milk, followed by pasteurisation, produced only slight flavour changes. Whole milk and chocolate milk fortified with ferripolyphosphate–whey protein complex exhibit good flavour properties (Jones *et al*. 1975; Douglas *et al*. 1981). Hegenauer *et al*. (1979c) showed that ultrasonic emulsification of milk fat prior to fortification greatly reduces lipid peroxidation.

2.4.14.7 Thiobarbituric acid (TBA) value

TBA test has been extensively applied to milk in which the absorbance of TBA reaction products correlated positively with organoleptic evaluation. King (1962) related the TBA value with flavour score of milk. Fortification with ferric chloride ($FeCl_3$), ferrous sulphate ($FeSO_4$) or ferric ammonium sulphate caused oxidation and higher TBA number (Demottt 1971; Edmonson *et al*. 1971; Kurtz *et al* 1973; Wang and King 1973; Hegenauer *et al*. 1979c; Douglas *et al*. 1981). Wang and King (1973) showed that the TBA value of ferric ammonium citrate fortified milk was as stable as control; and TBA value of ferrous sulphate fortified cow milk was four times higher than ferric ammonium citrate fortified counterpart. Hegenauer *et al*. (1979c) reported the relationship between TBA reactivity and temperature was very similar whether iron was added before or after heating period. They also reported that there was a slight increase in TBA reactivity associated with the lengthening of the time between addition of iron and pasteurisation. This increment was greater for ferrous sulphate than for chelated ferric iron. Hegenauer *et al.* (1979c) reported that iron (III) chelates of nitriloacetate and lactobionate catalysed less oxidation than ferrous salts.

2.4.15 Effect of environmental conditions and technological treatments

As iron supplemented dairy products could be used as food additives in different formulas, drinks, yoghurts and cheeses, different authors in the literature have described the effects of environmental conditions and various technological treatments on the characteristics of iron and the properties of iron supplemented dairy products.

2.4.15.1 Ionic environment

NaCl addition has no influence on the binding capacity of β-CN and sodium caseinate for iron (Baumy and Brule´ 1988; Gaucheron *et al*. 1997b). In the same way, iron remains bound to caseins even after addition of inorganic phosphate to iron supplemented caseinate. On the contrary, addition of citrate or EDTA induces iron transfer from caseins to these chelating agents (Gaucheron *et al*. 1996).

2.4.15.2 Acidification

During acidification of iron supplemented β-CN, caseinate and milk in the pH range 7-4, iron is never solubilised (Gaucheron *et al*. 1997b). Iron is probably strongly bound to Pser in the casein micelles and there is no association of iron to colloidal phosphate because it is not solubilised at the same time.

2.4.15.3 Ascorbic acid

Iron in the reduced state is an active prooxidant; and ascorbate, which could act a hydrogen donor, in synergism with iron, serves as a free radical initiating system as the following reaction:

$$Fe^{3+} + \text{ascorbate anion} \rightarrow Fe^{2+} + \text{ascorbate radical} + H^+$$

Moreover, ascorbate itself is an effective chelator (Rosenthal *et al*. 1993). However, Hegenauer *et al*. (1979a) indicated that the conversion of ascorbate to dehydroascorbate and of dehydroascorbate to dikegulonate occurs rapidly even in unsupplemented milk. Thus, iron supplementation may not affect materially the vitamin C content of stored milk.

2.4.15.4 Rennet coagulation

The initial oxidation state of iron is important because rennet coagulation parameters are differentially altered in the presence of ferrous chloride ($FeCl_2$) or ferric chloride ($FeCl_3$) (Gaucheron *et al*. 1997a). Increase in ferrous chloride ($FeCl_2$) concentration induces (i) big increase in clotting time, (ii) increase in the aggregation time and (iii) decrease in curd firmness. Increasing ferric chloride ($FeCl_3$) concentration induces a slight increase in clotting time, whereas the aggregation time increases and the curd firmness slightly decreases. Initial rates and the total amounts of caseinomacropeptide (CMP) released after rennet addition to supplemented skim milk containing 1.5 mM of iron are not different from those observed with non-supplemented skim milk. Thus, the primary phase of rennet coagulation is not affected by the presence of iron. These results suggested that (i) chymosin is probably not modified by iron, (ii) κ-CN, which has one to two Pser is unmodified and (iii) the preferential binding of iron to α_{s1}-CN does not affect the enzymatic hydrolysis of the κ-CN. Therefore, the secondary (aggregation of paracasein micelles) and tertiary (gel reticulation) phases could be altered by modifications of molecular interactions between paracasein micelles in the presence of iron. Reddy and Mahoney (1992) indicated that 0.2 mM ferric chloride ($FeCl_3$) does not significantly affect the rennet clotting time.

2.4.16 Iron and Maillard browning

Metal ions are known to activate the Maillard reaction (Kato *et al*. 1981), particularly the formation of carboxymethyllysine (Ahmed *et al*. 1986). By accelerating ascorbate oxidation, iron (III) also favours protein ascorbylation. Furthermore, iron is strongly chelated with glycated proteins (Qian *et al*. 1998) and can generate oxygen species, efficiently reacting with the amino acid residues located near the metal binding site (Amici *et al*. 1989). This oxidative process may explain the degradation of tryptophan in the presence of advanced Maillard products (Moreaux and Birlouez-Aragon 1997).

Lecle`rea *et al*. (2002) evaluated the deleterious actions of iron and ascorbate, namely lysine damage and tryptophan oxidation on whey-lactose samples incubated at 60ºC. A 3-folds increase in the accumulation rate of fluorescent advanced Maillard products (AMP) was observed in the presence of iron/ascorbate. The tryptophan degradation rate was 2.5-folds higher in the presence of iron-ascorbate.

2.4.17 Bioavailability

Bioavailability of a given nutrient from a diet (i.e. the release of the nutrient from the food, its absorption in the intestine and bioresponse) has to be taken into account. It is the level of the nutrient that should be present in the diet to meet the requirement. Bioavailability is a key determinant in iron fortification programs; identifying forms of irons which do not cause undesirable organoleptic changes in foods, however, are bioavailable and are subject to normal homeostatic conditions, is a high priority. Bioavailability includes the absorption and transport of nutrients to their relevant body tissues as well as their conversion into physiologically active species (Fairweather-Tait 1976; Godber 1990; Carpenter and Mahoney 1992).

Iron absorption from the diet is a complex process involving three steps: uptake from the intestinal lumen into mucosal cells, transit through the cell and release from the cells into the body. Although many factors are responsible for the onset of iron deficiency (Baynes and Bothwell 1990), the most likely cause of this nutritional problem in developing countries is poor iron bioavailability from the diet (Bothwell *et al*. 1989). Therefore, accurate assessment of iron bioavailability in diet is important.

2.4.17.1 Methods used to assess iron bioavailability:

- Haemoglobin repletion
- Plasma appearance (following >5-10 mg oral dose of iron)
- Fecal monitoring (chemical balance)
- Caco-2 *in vitro* system

(Fairweather-Tait *et al*. 2002)

The concentration of haemoglobin should be measured, even though not all anaemia is caused by iron deficiency. The prevalence of anaemia is an important health indicator and when it is used with other measurements of iron status, haemoglobin concentration can provide information about the severity of iron deficiency. However, measurements of serum ferritin and transferrin receptor provide the best approach for measuring the iron status of populations (WHO 2005)

2.4.17.2 Enhancers of non-haeme iron absorption

2.4.17.2.1 Ascorbic acid

Ascorbic acid enhances iron absorption, if eaten in diet together with iron. The absorption enhancing effect is more pronounced in inhibitory meals and meals which contain two main inhibitors of iron absorption viz. phytates and polyphenols (Cook and Monsen 1977; Siegenberg *et al*. 1991). Ascorbic acid acts by maintaining iron in a soluble bioavailable form as the luminal pH rises once the gastric contents enter the duodenum, since ferric form of iron is soluble only at acidic pH, however, the complex of iron and ascorbic acid is soluble over a wide pH range (Conrad and Schade 1968).

2.4.17.2.2 Organic acids

Gillooly *et al*. (1983) measured the iron absorption associated with 17 vegetable meals. All the vegetables associated with good iron bioavailability contained appreciable amounts of one or more of the organic acids citric, malic or ascorbic acids. The addition of citric, malic or tartaric acid to a rice-based meal improved iron absorption 2- to 4-folds (Ballot *et al*. 1987)

2.4.17.2.3 Animal tissues

Several animal tissues, including beef, chicken, fish, lamb, liver and pork, improve iron status both by supplying highly available haeme iron and by improving absorption from non-haeme iron pool (Cook and Monsen 1976; Lynch *et al*. 1989). Although the factors in animal tissue responsible for these beneficial properties remain poorly characterised, it has been suggested that peptides released during proteolytic digestion by pepsin in the stomach may increase the solubility of inorganic iron (Kane and Miller 1984; Hurrell *et al.* 1988; Slatkavitz and Clydesdale 1988).

2.4.17.3 Inhibitors of non-haeme iron absorption

2.4.17.3.1 Phytates

A large number of studies have demonstrated that phytates are the major iron absorption inhibitors in cereal foods such as wheat, oats, sorghum, unpolished rice and beans (Gillooly *et al*. 1983, 1984; Rossander-Hulthen *et al*. 1990; Hurrell *et al*. 1992). Monoferric phytate, which constitutes only a small proportion of the phytate in bran, is not inhibitory (Simpson *et al.* 1981), however, the formation of diferric and tetraferric phytate complexes in the gastrointestinal tract may render iron unavailable for absorption (Morris and Ellis 1982). Dephytinisation of food products seems to be a possible method to improve iron absorption (Hurrell *et al*. 1992).

2.4.17.3.2 Polyphenols

Disler *et al.* (1975a) reported that tea is a powerful inhibitor of iron absorption; this is primarily the result of its tannin content (Disler *et al*. 1975b). Polyphenols are thought to act through the formation of complexes between the hydroxyl groups of the phenolic compounds and iron molecules, rendering the iron unavailable for absorption.

2.4.17.3.3 Calcium

The addition of calcium in the form of milk or an inorganic salt to a meal reduces percentage non-haeme iron absorption in human beings. The addition of calcium and phosphate diminished percentage iron absorption while the addition of calcium alone had no effect. Hallberg *et al.* (1991) demonstrated that doses of calcium chloride (between 40 to 600 mg calcium) caused a dose-related reduction (upto 300 mg calcium) in non-haeme iron absorption from a meal of wheat rolls containing 10 mg native calcium and 3.8 mg iron.

2.4.17.3.4 Fiber

Components of fiber bind iron *in vitro*. However, there is little current evidence to suggest an important role for fiber in human studies. Enzymatic dephytinisation of bran almost completely removes the inhibitory effect (Hallberg 1987). Several purified fiber components have been tested for their effect on human iron absorption. Cellulose and pectins are not inhibitory. Ispagula and psyllium cause a mild reduction in absorption (Rossander 1987).

2.4.17.4 Nutrients with systemic effect on iron absorption

2.4.17.4.1 Vitamin A

A direct correlation between serum retinol and haemoglobin levels in women and children has been observed in several surveys (Mejia *et al*. 1977; Mohanram *et al*. 1977; Suharno *et al*. 1993). Anaemia associated with marginal vitamin A deficiency is characterised by a decrease in the serum iron concentration, total iron-binding capacity and transferrin saturation and with increased storage of iron in liver and spleen. Vitamin A deficiency impairs iron mobilisation from stores and has little influence on iron absorption (Amine *et al*. 1970; Mejia *et al* 1979) i.e. iron delivery to the developing erythroid cells appears to be impaired.

2.4.17.4.2 Ascorbic acid

There is direct interaction between ascorbic acid and non-haeme iron in the lumen of upper small bowel, which is not related to individual's ascorbic acid status. Ascorbic acid deficient human subjects have a defect in iron release from reticulo-endothelial cells. The administration of ascorbic acid to such individuals produces a rapid rise in the serum iron concentration (Wapnick *et al*. 1970).

2.4.17.5 Effect of other minerals on iron bioavailability

Fortification of food products with a trace element may impair utilisation of another trace element in the body because many elements interact with each-other and with other nutrients (Momcilovic and Kello 1979). High iron concentrations can negatively affect zinc absorption in adults when these trace minerals are given in solution (Whittaker 1998). However, when iron and zinc are given in a meal, this effect is not observed. Solomons (1986) postulated that the total amount of ionic species affects the absorption of zinc and that a total dose of >25 mg iron may produce a measurable effect on zinc absorption. However, it has been shown that if milk was fortified with physiological doses of iron, it would not interfere with the absorption and metabolism of zinc (Momcilovic and Kello 1979).

Cook *et al.* (1991) investigated the effects of calcium supplements (calcium carbonate, calcium citrate and calcium phosphate) on the absorption of dietary non-haeme iron and iron supplements. When calcium carbonate was taken without food, it did not prevent absorption of ferrous sulphate with doses of either 300 mg calcium and 9 mg iron, or 600 mg calcium and 18 mg iron. However, at the higher dose (600 mg calcium and 18 mg iron), calcium citrate and calcium phosphate significantly reduced absorption of iron by 49 and 62%, respectively. They concluded that regular calcium supplements taken with food inhibit iron absorption.

2.4.17.6 Iron availability in dairy products

Dairy products are good sources of minerals, vitamins and high quality proteins, however, contain almost no dietary iron. Therefore, dairy products could be used as a logical vehicle for iron fortification. The bioavailability of iron in dairy product depends on the iron sources (Theuer *et al*. 1973).

2.4.17.6.1 Milk

The wide consumption of milk by infants and children and its high nutrient density make it an attractive vehicle for iron fortification. Carmichael *et al*. (1975) studied the effect of milk and caseins on the absorption of supplemental iron in mice and chicks. They found that non-fat cow's milk and its constituent phosphoproteins did not inhibit iron absorption. Milk significantly increased the absorption of iron from ferric nitriloacetate chelate in the chicks. Ranhotra *et al*. (1981) studied bioavailability of a water soluble citrate phosphate iron complex in milk. They found that bioavailability of iron was as high as that of ferrous sulphate (99% vs 100%) and it was not affected by milk or its components. Park *et al*. (1986) compared the bioavailability of iron from goat and cow milk when the milk was fed to anaemic rats. Iron bioavailability of goat milk was greater than cow milk. Tsuchita *et al*. (1991) investigated iron bioavailability of ferric pyrophosphate by haemoglobin repletion assay. The iron sources were prepared from aqueous suspension of ferric pyrophosphate: mixed with skim milk and dehydrated (FP1), directly dehydrated (FP2); and FP2 mixed with skim milk and redehydrated (FP3). The relative bioavailability of FP1 was 100% that of $FeSO_4$ by slope ratio analysis. They concluded that bioavailability of ferric pyrophosphate was improved by mixing with skim milk and heat treating.

2.4.17.6.2 Cheese

A study on cottage cheese fortified with ferric ammonium citrate showed that bioavailability of iron was not affected relative to the time of iron addition during the manufacturing procedure (Wong *et al*. 1984). Zhang and Mahoney (1989) found that bioavailability of iron in cheddar cheese fortified with ferric chloride, iron-casein, ferripolyphosphate-whey protein and iron-whey protein complex was high (5, 8, 6 and 7%, respectively) and similar to ferrous sulphate (5%). They concluded that iron fortified cheese was a good source to increase human dietary iron intake.

2.4.17.6.3 Infant formulas

Stekel *et al.* (1986) investigated the bioavailability of iron added to infant formula. They found a higher range of iron absorption (5.9 to 11.3%) when ferrous sulphate was added in conjunction with ascorbic acid (100 mg/L) and reported that amount of milk fat, addition of carbohydrates or acidification would not affect iron absorption.

2.4.17.7 Iron binding milk proteins

Most of the iron added to milk binds to protein molecules. Bash *et al*. (1974) investigated the distribution of added iron polyphosphate in cow's milk and found that casein showed a greater binding affinity for iron than for phosphorus; about 85 to 95% of iron and 50-55% of phosphorus are bound to acid precipitated casein. Iron binding by casein is attributed to clustered phosphoseryl residues.

Ferrous salts are not usually recommended for fortification of high moisture products (Hegenauer *et al*. 1979b). Ferric iron binds rapidly to the casein phosphoserines and forms iron (III)-di-*o*-phosphoserine. Therefore, ferric iron has been suggested as a more suitable source for fortification of milk and milk products.

Saltman and Hegenauer (1983) reported that in cow milk, fat contained 3% of fortified iron while casein bound 75% of added iron. Human milk fat chelated significantly more iron than cow milk fat. Most of the total iron in infant formulas (casein or soy-based) is bound to soluble proteins (Galdi *et al*. 1987).

Lactoferrin and transferrin are also iron-binding proteins. Lactoferrin (Lf) occurs in three isomers: E_α binds iron; E_β and E_γ do not bind iron, however, show RNAase activity. These isoforms are very similar in isoelectric point, partial proteolytic peptide patterns and N-terminal amino acid sequence (Furmanski *et al*. 1989).

Shimazaki (1988) compared structure and iron-binding capacity of lactoferrin isolated from cow colostrum and cheese whey and found no difference in secondary and tertiary structures between the lactoferrins. However, the iron-binding capacity of cheese whey lactoferrin in human milk was resistant to trypsin digestion, whereas lactoferrin from bovine colostrum lost its iron-binding and anti-microbial activities after being exposed to trypsin.

2.4.17.8 Effect of processing on iron bioavailability

Effect of food processing to alter iron bioavailability is well established (Theuer *et al*. 1971, 1973; Jansuittivechakul *et al*. 1986). High heat treatments enhance the relative bioavailability of ferrous sulphate, sodium ferric pyrophosphate, ferric pyrophosphate and ferric orthophosphate supplemented in milk based infant formula (Theuer *et al*. 1973), soy isolate infant formula (Theuer *et al*. 1971), basal diets (Wood *et al*. 1978) and meat and meat products (Jansuittivechakul *et al*. 1986). Wood *et al*. (1978) reported that heat and pressure processing significantly enhanced the relative biological values for sodium ferric pyrophosphate and ferric pyrophosphate. Also typical retort conditions increase relative biological value of electrolytic iron and carbonyl iron by solubilisation and oxidation of the iron sources to the ferrous form (Clemens and Mercuric 1981). Theuer *et al*. (1973) also showed sterilisation of liquid based infant formulas increased the relative iron availability of ferric pyrophosphate from 75 to 125%.

2.5 Vitamin fortification of liquid milk

Milk is an oil-in-water emulsion, hence, the possibility exists to add vitamins in their oily form or use water dispersible forms. Ease of mixing has been identified as an advantage of using dry, water dispersible forms of the fortificant. However, the disadvantage of this is that vitamins are less stable in this form after addition to the milk as the protective coating gets dissolved leaving the vitamin susceptible to degradation (O'Brien and Robertson 1993). Addition of oily vitamin preparations is recommended after dilution and pre-homogenisation with a suitable quantity of milk.

2.5.1 Vitamin A fortification

National Nutrition Monitoring Bureau (NNMB 2003) estimated vitamin A intake in rural population across many states and determined that intake levels fluctuate from 30-60% of RDA of 600 mg/day. The above data validates the deficiency, which requires attention and needs to be compensated through fortification. The Department of Women and Child Development, Ministry of Human Resource, Government of India felt the necessity to fortify milk with vitamin A at a concentration level of 2000 IU/L of milk (Khanna 2005). Table 2.4 illustrates the current status of milk fortification with vitamins A in different countries.

Table 2.4: Mandatory fortification of liquid and dried milk in different countries

Country	Product	Vitamin A (IU/L)
Argentina	Fluid and dry milk (whole and skim)	2500
Brazil	Dry skim milk for complementary food programs	15000 - 25000*
Guatemala	Skim milk	2000-3000
Honduras	Milk	2000
Malaysia	Evaporated/unsweetened condensed milk	6700*
Malaysia	Sweetened condensed milk	6700*
Malaysia	Filled evaporated/filled condensed milk	6700*
Mexico	Sterilised low-fat milk	4000
Mexico	Pasteurised low-fat milk	4000
Mexico	Evaporated whole and low-fat milk	4000
Philippines	Filled evaporated/filled condensed milk	4866
USA	Fortified nonfat dry milk (reconstituted)	2115
USA	Evaporated milk	4225
USA	Evaporated skim milk	(4225)**
Venezuela	Dry milk powder	4000*

*IU/kg

**Figures in parentheses indicate that addition is optional.

2.5.1.1 Physiological significance of vitamin A

Vitamin A is an essential nutrient that is required in small amounts by humans for the normal functioning of the visual system, the maintenance of cell function for growth, epithelial cellular integrity, immune function and reproduction. Dietary requirements for vitamin A are normally provided as a mixture of preformed vitamin A (retinol), which is present in animal source foods and provitamin A carotenoids, which are derived from foods of vegetable origin and which have to be converted into retinol by tissues such as the intestinal mucosa and the liver in order to be utilised by cells. World Health Organisation (WHO) has defined vitamin A deficiency

(VAD) as tissue concentrations of vitamin A low enough to have adverse health consequences, even if there is no evidence of clinical xerophthalmia (WHO/NUT 1996).

2.5.1.2 Structure of retinol and its esters

Structure of retinol and its esters have been shown in figure 2.2, 2.3 and 2.4.

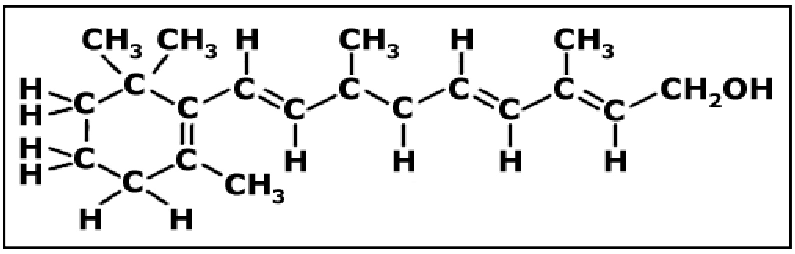

Figure 2.2: Structure of vitamin A (Retinol)

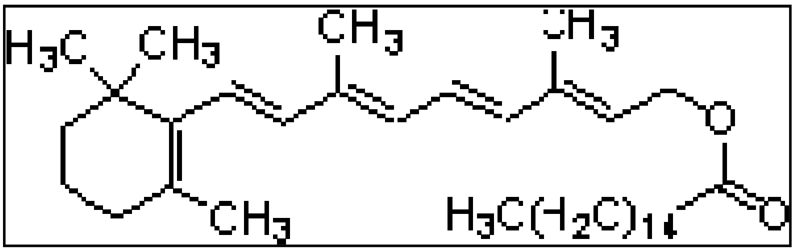

Figure 2.3: Structure of vitamin A palmitate (Retinyl palmitate)

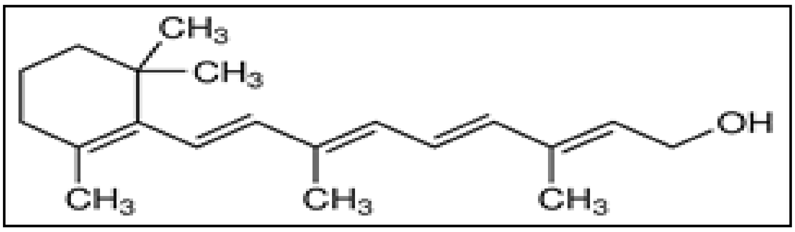

Figure 2.4: Structure of vitamin A acetate (Retinyl acetate)

2.5.1.3 Vitamin A requirements

Very low intakes, poor bioavailability of pro-vitamin A from the predominantly vegetarian diet and recurrent infections are thought to be the main reasons for widespread prevalence of vitamin A deficiency. Vitamin A is required for healthy vision and other physiological functions. Indian Council of Medical Research (ICMR) has recommended daily intake of 600 RE (retinol equivalents) of vitamin A for adult men and women (table 2.5).

Table 2.5: Recommended dietary allowance of vitamin A for Indians
(Retinol equivalents, RE, µg/d)

Group	RDA (RE, µg/d)
Adult men	600
Adult women	
Pregnant women	800
Lactating women	950
Infants 0-6 m	350
6-12 m	
Children 1-6 y	400
7-9 y	600
Adolescents 10-17 y	600
Source: ICMR 2009	

2.5.1.4 Vitamin A deficiency

Vitamin A deficiency (VAD) usually develops in an environment of ecological, social and economical deprivation, in which the key risk factors are a diet low in sources of vitamin A (i.e. dairy products, eggs, fruits and vegetables), poor nutritional status and a high rate of infections, in particular, measles and diarrhoeal diseases. Subclinical vitamin A deficiency is also associated with an increased risk of child mortality, especially from diarrhoea and measles (Allen *et al.* 2006).

In general, 3 main intervention strategies are currently in use to improve vitamin A status: (1) increasing the dietary intake of foods rich in vitamin A and provitamin A by changing dietary patterns, (2) periodic administration of large doses of vitamin A; and (3) fortification of vitamin A of one or more commonly consumed dietary items (Sommer 1995). Fortification of suitable foods with vitamin A is a well recognised approach to solve VAD problems in many parts of the world and particularly so in the developing nations (Favaro *et al*. 1991; Lee *et al*. 2000).

2.5.1.5 Safety issues

Adverse physiological effects have been associated with both acute hypervitaminosis A and chronic high intake. The routine consumption of large amounts of vitamin A over a period of time can result in a variety of toxic symptoms including liver damage, bone abnormalities and joint pain, alopecia, headaches, vomiting and skin desquamation (Allen *et al.* 2006).

2.5.1.6 Choice of vitamin A fortificant

The choice of a fortificant is largely governed by the characteristics of the food vehicle, as well as various technological, regulatory and religious considerations. As preformed vitamin A (retinol) is an unstable compound, it is esterified, usually with palmitic or acetic acid, to the more stable corresponding esters in commercial preparations. Retinyl acetate and retinyl palmitate, along with provitamin A (β-carotene), are thus the main commercial forms of vitamin A that are available for use as food fortificants. The intense orange colour of β-carotene makes it unsuitable for use as a fortificant in many foods, however, it is widely used to give an orange-yellow colour to margarines and beverages. Since vitamin A is fat soluble, it is easily added to fat based or oily foods. When the food vehicle is either dry or water based liquid, an encapsulated form of the vitamin is needed. Table 2.6 shows the commercially available forms of vitamin A.

Table 2.6: Commercially available forms of vitamin A, their characteristics and main applications

Product	Characteristics	Application (s)
Oily vitamin A acetate	Retinol ester of acetic acid which may be stabilised with antioxidants	Fortification of fat based foods, especially margarine and dairy products
Oily vitamin A palmitate	Retinol ester of palmitic acid which may be stabilised with antioxidants	Fortification of fat based foods, especially margarine and dairy products
Dry vitamin A palmitate or acetate	Vitamin A embedded in a water-soluble matrix (e. g. gelatin, gum acacia, starch) and stabilised with antioxidants	Fortification of dry products (i. e. flour and dry mix, beverage powders) and fortification of water-based foods

(Allen *et al.* 2006)

Bector and Rani (1998) developed a methodology for the fortification of milk with vitamin A without using homogeniser. It involved the preparation of a water dispersible vitamin A emulsion (containing butylated hydroxyl anisole (BHA), Tween 80, sodium citrate, monopotassium dihydrogen phosphate and sodium alginate) which could be directly added to milk and mixed uniformly by just physical means. Vitamin A content of fortified toned milk was, however, significantly affected on boiling and sterilisation, and on exposure to sunlight and fluorescence light. The cost of fortification of toned milk with vitamin A at level of 2,000 IU was less than two paise per litre.

2.5.1.7 Stability

Vitamins are sensitive to heat, light and humidity, as well as oxidising and reducing agents to different degrees. Recent advances in technology have enabled the production of commercial forms of vitamins with improved stability and compatibility with other micronutrients. Nutrients naturally present or added to liquid milk are fairly stable during processing. Most vitamins and minerals show retention of 70 to 100% after a single common industrial heat treatment. However, repeated heat treatments can result in extensive losses. The stability of most nutrients in liquid milk during storage is also good.

Vitamin A is quite stable when heated to moderate temperatures in the absence of oxygen and light. Overall loss of activity during anaerobic heating may range from 5-50%, depending on time, temperature and nature of carotenoids (Tannenbaum *et al.* 1985). In the presence of oxygen and light, there can be extensive loss of vitamin A activity through oxidation. The presence of trace metals accelerates this reaction. In dehydrated foods, vitamin A and provitamin A are highly susceptible to loss by oxidation. The extent of this loss depends on the severity of the drying process, protection provided by packaging materials and conditions of storage. Vitamin A in its pure form is unstable in presence of mineral acids but stable in presence of alkali (Labuza *et al*. 1970).

Maguer and Jackson (1983) assessed the content and stability of vitamin A in fortified fluid milks. Destruction of the vitamin by pasteurisation or direct ultra-high temperature treatment was minimal. The vitamin remained relatively stable up until and beyond the coded pull dates of the pasteurised (12 days) and ultra-high temperature processed (3 months) milk samples stored at 4 and 20ºC, respectively. Storage of 2% ultra-high temperature processed milk at 35ºC adversely affected its vitamin stability, notably after 12 weeks of storage. Cartons of the 2% ultra-high temperature processed white and chocolate milks that had been stored either at 20 or 35ºC for varying periods, once opened, may be kept for 12 days at 4ºC without appreciable decline in vitamin A content.

Medrano *et al.* (1994) studied the effect of microwave heating for 2-4 minutes on retinol content of milk and found that in whole milk, retinol content was not modified after microwave heating for 2-4 minutes, in low fat milk, microwave heating for 2 minutes lowered retinol content (91.7% retention) and in skim milk, retinol was not detected before or after microwave heating.

Bector and Rani (1998) reported some loss of vitamin A during boiling and sterilisation. They also observed vitamin A losses on exposure to sunlight and fluorescent light. There was no substantial loss in vitamin A content of fortified toned milk at refrigerated temperature (5-7ºC) for a storage period of 48 hrs. However, beyond this period, there was loss in vitamin A content and it increased as the period of storage increased. A loss of 3.5% of vitamin A content of fortified toned milk was observed after a storage period of 72 hrs which increased to 10.2% at the end of 96 hrs. Wishner (1964) stated that loss of vitamin A activity results from an opening of the β-ionone ring portion of the vitamin A molecule.

Gaylord *et al*. (1986) quantified retinyl palmitate in milk samples exposed to fluorescent light. Effect of compositional factors was determined by comparing rates of loss of vitamin A in milks with different amounts of milk fat. Upon exposure to fluorescent light, rate of vitamin A loss was lower in whole milk than in skim milk. Increasing light intensity increased the loss of vitamin A. Increased fat percent had a protective effect on vitamin A. More retinyl palmitate was retained in 2% and whole milks than in skim milk after exposure to fluorescent light at 1614 lux (150 ft-c). Decreased stability of light sensitive vitamins in low fat milks could be due to increased light penetration. Rate of retinyl palmitate loss increased with increased light intensity.

Haisman *et al*. (1992) observed that cardboard/polyethylene laminate cartons protect better than plastic containers both the nutrients and the taste and flavour of milk against the deleterious effects of light.

Saffert *et al*. (2008) evaluated the effect of package light transmittance on the vitamin content of fortified UHT low-fat milk. Milk was stored under light with an intensity of 700 lux in polyethylene terephthalate (PET) bottles with varying light transmittance to monitor the changes in the vitamin A content over a storage period of 12 weeks at 23°C. In clear PET bottles, a reduction of 93% of the initial content was observed. In all pigmented PET bottles, the vitamin retention was only slightly higher; the losses ranged between 70 and 90% for vitamin A. In the control sample stored in dark, a 16% loss could be observed for vitamin A.

Coulter and Thomas (1968) pointed out that vitamin A is more stable in whole milk products than in low fat or skimmed milk products, presumably because of natural antioxidants present in milk fat**.** Borenstain (1979) and Ottaway (1993) both have reported that vitamin A (and also β-carotene) added to foods is sensitive to oxidative damage. In the form of retinol, vitamin A is more labile than its ester form; for this reason, vitamin A esters are usually used for food fortification. As high temperatures may be used in the manufacture of fortified foods, measures must be taken to minimise losses from thermal degradation.

Bartholomew and Ogden (1990) studied the comparative stability of naturally occurring vitamin A in milk and that added externally. They found that vitamin A added to milk is more light sensitive and degraded more rapidly than indigenous vitamin A when concentrated oil solutions of vitamin A and emulsifier were used for fortification.

Micronutrients added to milk, thus can be destroyed during the normal thermal processing and storage of milk. To compensate for these losses, an appropriate overage of each micronutrient must be added during fortification.

2.5.1.8 Methods for vitamin A analysis

Retinol, its derivatives and metabolites have been determined by several techniques. The analytical techniques that were commonly used in the past were column chromatography, thin-layer chromatography (TLC) and gas liquid chromatography. However, the high-performance liquid chromatographic (HPLC) technique has proven to be by far the method of choice for the determination of retinoids. Table 2.7 illustrates different HPLC methods used for vitamin A analysis.

Table 2.7: HPLC methods for analysis of vitamin A in different matrices

Matrix	Column	Conditions	Reference (s)
Fluid dairy products	NOVA-PAK C_{18}, stainless steel, 3.9 mm x 15 cm	Isocratic; mobile phase: methanol:water (95:5); flow rate= 0.8 ml/minute; volume injected= 20 µl; UV detector, absorbance at 325 or 313 nm	Zahar and Smith (1990)
Milk	HS-5 Silica, 125 x 4.0 mm	Isocratic; mobile phase: heptane:isopropyl alcohol (94:6); flow rate= 1 ml/minute; fluorescence detector, excitation wavelength (344 nm), emission wavelength (472 nm)	Jensen (1994)
Milk	Spherisorb ODS2, 25 x 0.46 cm (5 µm)	Isocratic; mobile phase: methanol:water (98:2); column temperature: 30ºC; flow rate= 1 ml/minute; UV detector, absorbance at 325 nm	Medrano *et al.* (1994)
Cheese	LiChrosorb Si 60, 250 x 4.6 mm	Isocratic; mobile phase: hexane:isopropyl alcohol (99.8:0.2); flow rate= 2 ml/minute; UV detector, absorbance at 325 nm	Marsh *et al*. (1994)
Liver, liver products, infant foods	Spherisorb SW, 100 x 2 mm (3 µm)	Isocratic; mobile phase: isooctane:isopropyl alcohol (98.75:1.25); flow rate= 0.4 ml/minute; UV detector, absorbance at 325 nm	Brinkmann *et al.* (1995)
Human milk	Grom-Sil-CN-2PR, 250 x 4.6 mm, (5 µm)	Isocratic; mobile phase: hexane:isopropyl alcohol (98:2); flow rate= 2 ml/minute; fluorescence detector, excitation wavelength (344 nm), emission wavelength (480 nm)	Strobel *et al.* (2000)
Corn flakes (vitamin A palmitate)	Normal phase silica column (Spherisorb S5W 4.6 x 150 mm)	Isocratic; mobile phase: hexane:diethyl ether (99:1); flow rate = 1 ml/minute; UV detector, absorbance at 327 nm	Kim *et al*. (2000)
Milk products (applicable to raw and pasteurised skim, reduced fat and whole milks)	Guard column: SupelGuard Analytical column: Supelcosil LC-18; 250 x 4.6 mm	Isocratic; mobile phase: methanol:water (95:5); ambient temperature; flow rate: 1.5 ml/minute; UV detector, absorbance at 325 nm	Wehr and Frank (2004)

2.5.1.9 Vitamin A bioavailability

In vitro models based on human physiology have been developed as simple, inexpensive and reproducible tools to predict the bioavailability of different food components. Herrero-Barbudo *et al*. (2009) assessed the applicability of an *in vitro* digestion model to estimate the bioaccessibility of fat-soluble vitamins contained in commercially available liquid milk. The authors reported that *in vitro* results were consistent with *in vivo* observations supporting the potential applicability and predictive value of the *in vitro* approach to assess the bioavailability of fat soluble vitamins from dairy matrices.

2.6 Multiple fortification

Multiple fortification of foods is a possible way of addressing deficiencies of two or more micronutrients at the same time in a cost-effective manner (Darnton-Hill and Nalubola 2002) and does not pose any risk of substantial losses or any deteriorative effect during storage or baking on native mineral contents in whole wheat flour, a highly preferred food carrier for fortification of minerals in the developing world (Akhtar *et al*. 2009).

Multiple micronutrient fortification can be more effective in improving nutritional status than fortification with single key micronutrients; therefore, the multiple micronutrient fortification of appropriate food vectors, including milk, is of interest from the nutritional standpoint. A number of trials have evaluated the efficacy of foods and beverages as vehicles for multiple fortification. In South Africa, fortification of biscuits with iron, β-carotene and iodine improved the status of all of these nutrients in schoolchildren (Van Stuijvenberg *et al.* 2001).

2.6.1 Association between vitamin A and iron

Lack of vitamin A and iron in humans and experimental animals may result in anaemia (Frank 1940; Wagner 1940). Iron deficiency is epidemiologically associated with vitamin A deficiency (Mejia 1977). Vitamin A deficiency inhibits iron utilisation and accelerates the development of anaemia (Hogdes *et al.* 1978). Vitamin A fortification improves the haematological indices of population. This is a well-established fact that vitamin A (retinol) is mobilised from the liver by an iron-dependent enzyme, however, more recently, experimental studies have suggested that in case of iron deficiency, the vitamin is trapped in the liver and thus may be less accessible to other tissues and organs (Rosales *et al.* 1999). Therefore, combined supplementation of vitamin A and iron may have positive influence on health (Schultink and Gross 1996).

There are some evidences from both experimental animal models and human studies which suggest that vitamin A deficiency may exacerbate anaemia through impairment of iron metabolism (Semba and Bloem 2002). These micronutrient interactions strongly argue for multiple micronutrient fortification (Wegmuller *et al*. 2006).

The simultaneous addition of vitamin A and C to corn flakes, produced a 3.6 times increase in iron absorption compared to no vitamin addition and a 2-times increase compared to the absorption from the cereal fortified with only one of the vitamins. This implies that both vitamins, at the concentrations used in commercially available corn flakes, were capable of enhancing iron absorption at the same level, thus increasing iron absorption approximately 2 times when administered separately and almost 4 times when administered together. Addition of vitamins A and C at certain concentrations can act synergistically as enhancers of reduced iron absorption in fortified corn flakes (Garcia-Casal *et al*. 2003).

Layrisse *et al*. (1997) observed that iron absorption from bread was increased to about 100% when 1,000 international units of this vitamin A reacted with 5 mg of iron as ferrous fumarate plus one mg of food iron. Their results suggest that vitamin A binds with iron liberated during digestive process and acts as a quelating agent, which keeps iron soluble in the intestinal lumen and thus prevent the inhibitory effect of polyphenols and phytates on non-haeme iron absorption.

2.7 Fortification level

In developed countries, regulation of fortification is currently receiving more attention than technologies involved. This is because there is a legitimate fear of over fortification as manufacturers seek to use fortification as a marketing tool. The regulatory concerns associated with the labeling of a product and nutrient addition are of utmost importance to food technologists (Giese 1995).

The fortification policy set out by the Food and Drug Administration (FDA) in Title 21 CFR 104.20 (CFR 1984) seeks to establish a uniform model or principles for the rationale addition of nutrients to foods. These guidelines attempt to achieve and maintain a desirable level of nutritional quality in nation’s food supply, discourage random fortification that could result in over- or under-fortification to create nutrient imbalances, discourage deception or misleading claims and indiscriminate addition of nutrients to inappropriate foods such as fresh produce, meat, sugars, snack foods, candies and carbonated beverages (Giese 1995).

The levels at which nutrients are added to milk depend on a number of factors, including levels of milk consumption and nutritional requirements of the target population; the effect of added nutrients on the functional or sensory (odour, flavour and colour) characteristics of milk; and the stability of the nutrients during processing and storage of milk (www.nutritionimprovement.com, accessed on August 17 2010).

According to Appendix O of the Grade “A” PMO (2007 Revision), vitamin A fortified fluid dairy products should contain not less than 100% and not more than 150% vitamin A of the required values or label claims” (USPHS 2007). Tolerable upper intake levels (TU) for vitamin A and iron are 3000 µg/day and 45 mg/day for adults, respectively (FAO/WHO 2004).

The fortification level of 5000 IU vitamin A/1000 g dry skim milk is used globally, based on a daily intake of 40-80 g dry skim milk/subject. In United States, addition of vitamin A to whole milk is optional, but when added it must be present at a level of 2000 IU/quart (0.946 litre).

To meet label claims within a realistic shelf life, manufacturers must study the behaviour and kinetics of nutrient degradation thoroughly. To make correct claims about the nutrient content of a product on its label, the amount of the added nutrient should actually be more than that amount stated or declared on the label. The difference between the formulated and the declared levels is known as overage.

$$\text{Overage} = \frac{\text{(amount of nutrient present in the product - amount declared on the label)}}{\text{amount declared on the label}} \times 100$$

The overage will vary according to the inherent stability of the nutrients, the conditions under which the food is prepared and packaged and the anticipated shelf life of the product. Thus, for more labile or unstable nutrients, such as vitamin A, generally high overages are required.

2.8 Cost

The cost of milk fortification is limited to the cost of the micronutrients to be added and the cost of monitoring the quality of fortification. Changes needed in the usual milk production line are minimal. The cost of raw materials to fortify pasteurised milk with 5000 IU of vitamin A, 500 IU of vitamin D and 100% of the USRDA of vitamin E, C, B_1, B_2, B_6, B_{12}, niacin and folate is estimated at US$ 1.59 per 1000 litres. This amounts to less than 1% of the average wholesale price of liquid milk in USA ($ 0.29/litre).

CHAPTER –3

Materials and Methods

3. Materials and Methods

The present study was carried out for development and evaluation of vitamin A and iron fortified milk. Toned milk (cow milk:buffalo milk 1:1) was fortified with vitamin A and iron singly and in combination. Screening and optimisation of the type and level of fortificants was done on the basis of sensory scores, pH, acidity and TBA value. The physico-chemical properties (viscosity, curd tension, rennet coagulation time, alcohol stability, colour and % dry sediment) and heat stability of fortified milk were studied. Stability of added micronutrients in fortified milk was studied during different heat treatments, storage conditions and in different packaging materials. Distribution of the added iron was estimated among different milk fractions. Ultracentrifugation of milk was carried out to evaluate the fractionation behaviour of iron in colloidal and aqueous phases of milk. Effect of added micronutrients on electrophoretic behavior of milk proteins (by Urea-PAGE and SDS-PAGE) was also studied. Effect of fat content on quality characteristics (organoleptic properties, viscosity, curd tension and oxidative stability) of fortified milk was studied. The bioavailability of added nutrients was detected *in vivo* (Wistar albino rats) and *in vitro* (simulated gastro-intestinal tract conditions). The study was carried out in Dairy Chemistry Department, National Dairy Research Institute, Karnal, Haryana, India.

This chapter deals with the materials and methods used for the preparation of fortified milk. The physico-chemical and analytical methods used for product analysis are also discussed.

3.1 Chemical reagents

All the reagents used were of “AR” grade unless otherwise specified.

3.1.1 Iron salts and vitamin A esters used for fortification

All the iron salts and vitamin A esters used were of food grade and were used according to availability. The iron salts used for screening were ferrous sulphate heptahydrate, BioFer (microencapsulated ferrous sulphate heptahydrate: liposome), ferrous gluconate hydrate, ferrous lactate hydrate, ferric chloride hexahydrate, ferric ammonium citrate (brown), ferric ammonium citrate (green), ferric pyrophosphate soluble and monosodium ferric EDTA. Vitamin A esters used for screening were vitamin A palmitate and vitamin A acetate.

Both the vitamin A esters, ferrous sulphate heptahydrate, ferrous gluconate hydrate, ferrous lactate hydrate, ferric chloride hexahydrate, ferric ammonium citrate brown, ferric ammonium citrate green have been granted GRAS status (part 184, title 21 of Code of Federal Regulations 1984)

Monosodium ferric EDTA (NaFeEDTA) has been reviewed as food additive by International Nutritional Anaemia Consultative Group (INACG) and has been provisionally accepted by the Joint FAO/WHO Expert Committee on Food Additives (1993).

Ferric pyrophosphate (FPP) soluble is also known as sodio-citro-ferric pyrophosphate and it is a chemical combination of ferric pyrophosphate and sodium citrate. Both ferric pyrophosphate and sodium citrate are affirmed as GRAS by USFDA (part 184, title 21 of Code of Federal Regulations 1984). FPP soluble has been used in the treatment of iron deficiency anemia (dose 120 to 500 mg). FPP soluble is a ferric iron chelate which was used in the United States as a hematinic in the early 1900s but is no longer prescribed for treatment of anaemia. Recently, related research on iron compounds showed that FPP soluble is uniquely compatible with lipid emulsions and may be a well tolerated source of iron intravenously when administered as an additive to haemodialysates and total parenteral nutrition admixtures (Gupta *et al*. 1999). The iron core of FPP soluble is tightly chelated to citrate and pyrophosphate ligands. Unlike the similarly named ferric pyrophosphate, which is essentially insoluble in aqueous solutions, FPP soluble is very soluble in aqueous solutions over a wide range of pH (pH 2-8). Food grade FPP soluble is quite inexpensive and is allowed as an iron fortificant in Europe, however, currently has no similar applications in United States (Zhu *et al.* 2009).

Cold water soluble vitamin A acetate (potency 325000 IU/g) was procured from DSM Nutritional Products India Pvt. Ltd., Mumbai, India and cold water soluble vitamin A palmitate (potency 250000 IU/g) from Piramal Healthcare Ltd., Mumbai, India.

Ferric ammonium citrate (green and brown), ferric chloride hexahydrate, monosodium ferric EDTA were purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India. Ferrous gluconate hydrate and ferrous lactate hydrate were purchased from Sigma Aldrich, St. Louis, MO, USA; ferrous sulphate heptahydarte from Rankem, RFCL, Ltd., Delhi, India. Ferric pyrophosphate soluble was supplied by Shanpar Industries Pvt. Ltd., Vadodara, Gujarat, India and BioFer (microencapsulated ferrous sulphate heptahydrate) from Zenica Bioplus Pty Ltd., Balwyn North, Victoria, Australia.

3.1.2 Chemicals

Disodium hydrogen phosphate, calcium chloride, sodium chloride, sodium hydroxide, M-17 broth, MRS broth, trichloroacetic acid, glycine, ammonium persulphate (APS), starch soluble and oxalic acid were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Chloroform and TEMED (N, N, N’ N’ tetramethylenediamine) were procured from Laboratory Reagents and Fine Chemicals, Loba Chemie, Mumbai, India; cholesterol, citric acid, phenolphthalein indicator, potassium dichromate, acrylamide, tris (tris (hydroxymethyl) aminomethane), sodium bicarbonate, potassium chloride, manganese sulphate, calcium carbonate, butylated hydroxyl toluene (BHT), *o*-phosphoric acid (HPLC grade) from Qualigens Fine Chemicals, Mumbai, India; n-hexane, ethyl acetate, pyrogallol, hydrochloric acid, glacial acetic acid, sodium thiosulphate, glycerol, acetonitrile (HPLC grade), water (HPLC grade) from Rankem, RFCL Ltd., New Delhi, India; retinol (HPLC standard), apple pectin powder, Tween 60, Tween 80, 2-thiobarbituric acid, 5-hydroxy methyl furfural (HMF), egg lecithin, bromophenol blue, Coomassie Brilliant Blue R-250, glucuronic acid, glucoseamine hydrochloride, agar, α-amylase from hog pancreas (activity 53.7 U/mg), mucin (type III) from porcine pancreas, pepsin from porcine gastric mucosa (crystalline, lyophilised powder, activity 2950 units/mg), albumin from bovine serum, bile salts, taurocholic acid sodium salt hydrate, pancreatin from porcine pancreas, lipase from porcine pancreas (activity 427 units/mg, lyophilised powder), colipase from porcine pancreas (lyophilised powder), phospholipase A_2 from porcine pancreas and cholesterol esterase from porcine pancreas (lyophilised, activity 35 units/mg) from Sigma Aldrich, St. Louis, MO, USA; diethyl ether, sodium dihydrogen phosphate, sodium dodecyl sulphate (SDS) and methanol (HPLC grade) from Sisco Research Laboratories (SRL) Pvt. Ltd., Mumbai, India; potassium hydroxide, nitric acid, sodium thiosulphate, chloroform from Thermo Fisher Scientific India Pvt. Ltd., Delhi, India; sodium alginate and urea from Thomas Baker (Chemicals) Ltd., Mumbai, India; ethyl alcohol from Jiangsu Huaxi International Trade Company Ltd., Jiangsu, China; N,N'-methylene-bis-acrylamide, sucrose, cellulose, magnesium chloride, zinc sulphate, isopropyl alcohol and cyanocobalamin (vitamin B_{12}) from s.d. Fine Chem. Ltd. (SDFCL), Mumbai, India; glucose, copper sulphate, ammonium hydroxide and ammonium chloride from Glaxo Laboratories, Mumbai, India; skim milk powder from Modern Dairies Ltd., Karnal, Haryana, India; Megazyme acetaldehyde assay kit from Prolab Marketing Pvt. Ltd., New Delhi, India; dialysis membrane (molecular weight cut off 12000 Da) from Orange Scientific, Braine-l'Alleud, Belgium; haemoglobin estimation kit (cyanomethaemoglobin standard and Drabkin reagent) from JM Techno Chem., Haryana, India; riboflavin, tocopheryl acetate, biotin, nicotinic acid, pyridoxine hydrochloride, choline chloride, cobalt chloride, DL-methionine and meso-inositol from Central Drug House Pvt. Ltd., New Delhi, India; potassium dihydrogen phosphate and vitamin K_3 from Titan Biotech. Ltd., Bhiwadi, Rajasthan, India; potassium iodide from Sarabhai M. Chemicals, Baroda; furosine hydrochloride (HPLC standard) from Polypeptide Group, Strausberg, France; Meito rennet produced from *Mucor pusillus* var. *lindt* from Meito Sangyo Co. Ltd., Tokyo, Japan; protein molecular weight markers (205 to 3.5 kDa, GeNei^{TM}) from Merck Biosciences, Bengaluru, India; ferritin and transferrin ELISA kits from MyBioSource, San Diego, California, USA were used. For iron microencapsulation, soya lecithin was purchased from Himedia Laboratories Pvt. Ltd. Mumbai, India, Srinidhi Enterprises, Indore, India, Sonic Biochem, Indore, India and Sigma Aldrich, St.

Louis, MO, USA; egg lecithin from Sigma Aldrich, St. Louis, MO, USA and polyglycerol monostearate (PGMS) from Oleon, N. V., Vaarstraat, Belgium. Dahi culture NCDC 167 (mixed culture: *Lactococcus lactisssp*. *lactis*, *Lactococcus lactis ssp*. *diacetyllactis*, *Lactococcus lactis ssp*. *cremoris, Leuconostoc spp.*), yoghurt starter culture NCDC 074 (*Streptococcus thermophilus*) and NCDC 009 (*Lactobacillus delbrueckii* subsp. *bulgaricus*) were procured from National Collection of Dairy Cultures (NCDC), National Dairy Research Institute, Karnal, Haryana, India.

3.2 Apparatus and glassware

All volumetric flasks, pipettes and burettes were class "A". Burette (50 ml), funnels (small and large), graduated centrifuge tubes (15 ml low actinic), measuring cylinder (10, 50, 100, 250, 500 and 1000 ml), silica crucible, volumetric flasks (5, 10, 50, 100, 250, 500 and 1000 ml, low actinic and/or clear) and heat stability tubes were purchased from The Laboratory Glass Co. (LABCO), Ambala, India. Petridishes, staining box and gel scoop were procured from Tarsons Products Pvt. Ltd., Kolkata, India; parafilm from Sigma Aldrich, St. Louis, MO, USA; glass test tubes from Borosil India Ltd., Mumbai, India and filter papers (Whatman filter paper no. 1, 4, 41 and 42) from Whatman International Ltd., Kent, England.

3.3 Equipments

1. Autopipettes (10-100 µl and 100-1000 µl): Tarsons Products Pvt. Ltd., Kolkata, India
2. Syringe driven filter unit (33 mm, 0.22 µm pore size): Millex, Millipore, Billerica, Massachusetts, USA
3. Hamilton microlitre syringe (25 µl capacity) (for manual injections): Hamilton Company, Nevada, USA
4. Analytical scale: Sartorius BP2215, Goettingen, Germany
5. Cream separator: Kamdhenu, KD-60E, New Delhi, India
6. pH meter: PHAN, Labindia Instruments Pvt. Ltd., Maharashtra, India
7. High precision water bath: PolyScience, Illinois, USA
8. Shaking water bath: REMI Laboratory Instruments, Mumbai, India

9. Oilbath: Akash Deep Scientific, New Delhi, India
10. Laboratory shaker: Spinix, Vortex shaker, Tarsons Products Pvt. Ltd., Kolkata, India
11. Magnetic stirrer: Spinot, Tarsons Products Pvt. Ltd., Kolkata, India
12. Vacuum filtration assembly (1000 ml): Schott Duran, Riviera, Wertheim, Germany
13. Vacuum pressure pump: Millipore (India) Pvt. Ltd., Bengaluru, India
14. Ultrasonicator: SONICS, Vibra Cell, Model VCx750, Sonics and Materials Inc., New Town, USA
15. Hot plate: Advanced Technocracy Inc., Ambala, India
16. Hot air oven: Tempo Instruments and Equipments (I) P. Ltd., Mumbai, India
17. Muffle furnace: Narang Scientific Works Pvt. Limited, Delhi, India
18. BOD Incubator: Narang Scientific Works Pvt. Limited, Delhi, India
19. Droplet generator: CIPHET, Ludhiana, Punjab, India
20. Airless paint sprayer: Wagema Professional Quality, Pretoria, South Africa
21. Pycnometer: The Laboratory Glass Co. (LABCO), Ambala, India
22. Ostwald's U-tube viscometer: The Laboratory Glass Co. (LABCO), Ambala, India
23. Brookfield's viscometer: Visco Star Plus, Fungilab, Barcelona, Spain
24. Hunter Lab Colorflex Colorimeter: Hunter Associates Laboratory Inc., Reston, USA
25. Laboratory centrifuge (Model R8C): REMI Laboratory Instruments, Mumbai, India
26. Refrigerated centrifuge: Kubota Corporation, Gyeonggi-do, South Korea
27. Ultracentrifuge: Beckman L8-80M centrifuge 55.1, Ti rotor, Beckman Coulter, Sydney, Australia

28. UV-VIS Spectrophotometer: double beam, SPEORD 200 Analytik Jena, Jena, Germany
29. Rotary vacuum evaporator, Buchi type: Metrex Scientific Instruments Pvt. Ltd., New Delhi, India
30. Freeze dryer: Daihan Lab Tech India Pvt. Ltd., Hyderabad, India
31. Inverted light microscope: Nikon Eclipse Ti, Tokyo, Japan with a fitted digital camera (Nikon Digital Sight, Japan). Nikon Basic Research Imaging Software (v 3.1) for size measurement.
32. HPLC system and accessories: Waters High Pressure Liquid Chromatography 515 (Milford, Massachusetts, USA). It consisted of pump control module II with two Waters 515 HPLC pumps; rheodyne manual injector, temperature controlled column compartment and Waters 2998 photodiode array detector, Sample loop 20 µl. Chromatograms were analysed using Empower 2 software.
33. Atomic absorption spectrophotometer (AAS): AA-7000, Shimadzu, Tokyo, Japan, flame mode, air-acetylene flame (temperature 2300ºC with flow rate 1.5 L/minute)
34. Rancimat: Metrohm Rancimat Model 743, Herisau, Switzerland
35. Electrophoresis unit: Sigma Aldrich-Techware, Milwaukee, Wisconsin, USA: mini slab gel electrophoresis unit (8x7 cm). A large buffer reservoir (1 no.), rubber gaskets (2 nos.) and platinum electrodes (2 nos.), glass plates notched and rectangular (2 sets), 6 well teflon combs 0.5 mm (2 nos.) and clamps.
36. Densitometer: ImageAide software for Windows, Spectronics Corporation, Westbury, New York, USA.
37. Texture analyser: TA-XT2, Stable Micro Systems Ltd., Godalming, UK
38. Digital colony counter: Optics Technology, Delhi, India
39. Automated ELISA plate reader: CPC Diagnostics Pvt. Ltd., Chennai, India

3.4 Collection of milk samples

Fresh cow milk and buffalo milk were collected from the herd of cows and buffaloes maintained in the cattle yard of National Dairy Research Institute, Karnal, Haryana, India after the morning and evening milking and mixed in equal proportions (1:1). Cream was then separated with the help of cream separator. Milk was standardised to 3% fat and 8.5% solid-not-fat (SNF) using Pearson's square method.

3.5 Addition of iron salts and vitamin A esters to milk

3.5.1 Addition of iron salts

The iron content of salts was calculated from their molecular weight for ferrous sulphate heptahyrate, ferric chloride hexahydrate, ferrous gluconate hydrate and ferrous lactate hydrate while for ferric ammonium citrate brown and green, BioFer, ferric pyrophosphate soluble and monosodium ferric EDTA, iron content as specified by the suppliers was taken into account. Amount of salt to be added was then calculated for increasing the iron content of milk by 15, 20 and 25 mg per 1000 ml as shown in the table 3.1:

$$\text{Iron content} = \frac{\text{Molecular weight of iron salt}}{\text{Molecular weight of iron}} \times \text{level required}$$

3.5.2 Addition of vitamin A esters

Cold water soluble vitamin A acetate and vitamin A palmitate were used for fortification of toned milk (cow milk:buffalo milk 1:1) at the level of 2000 IU, 2500 IU and 3000 IU per litre. One International unit (IU) of vitamin A is equivalent to 0.33 µg vitamin A. To prepare stock solutions of 10000 IU/ml vitamin A, 307.7 mg vitamin A acetate was dissolved in some water and volume was made up to 10 ml; for vitamin A palmitate, 400 mg was dissolved in some water and volume was made up to 10 ml. Calculated amounts of these stock solutions were added to milk to achieve the desired levels of fortification.

Table 3.1 Quantity of iron salts needed for required level of fortification

Iron salt	**Molecular weight**	**Iron salt requirement (mg/100ml of milk) Iron concentration (ppm)**		
		15	**20**	**25**
Ferrous sulphate heptahydrate	278.02	7.47	9.64	12.45
BioFer (microencapsulated ferrous sulphate heptahydrate)	Fe content: 5.49% w/w; 6.53% w/v	11.98	14.57	18.21
Ferrous gluconate hydrate	446.16	11.98	15.98	19.98
Ferrous lactate hydrate	233.99	6.28	8.38	10.48
Ferric chloride hexahydrate	270.30	7.26	9.78	12.22
Ferric ammonium citrate (brown)	279.99; Fe content: 17-18.5%	8.82	11.76	14.70
Ferric ammonium citrate (green)	279.99; Fe content: 14-16%	10.71	14.28	17.85
Ferric pyrophosphate soluble	498.77; Fe content: 13.12%	11.43	15.24	19.05
Monosodium ferric EDTA	367.1; Fe content: 12-14%	12.48	16.66	20.82

3.6 Technology of fortification

Toned milk was taken in clean and dried conical flasks and the calculated amounts of iron salts, vitamin A esters (singly and in combination) were weighed and added. Addition of salt was accompanied by thorough mixing for 10 minutes with the help of magnetic stirrer for the complete dissolution of fortificants. After addition of salts, the milk samples were pasteurised at 63ºC for 30 minutes followed by cooling to 4ºC in an ice bath. Sensory analysis of milk samples was conducted after keeping the pasteurised samples for 2 hrs under refrigerated conditions (4-7ºC). Figure 3.1 illustrates the major steps involved in fortification of liquid milk.

Bulk whole milk
↓
Mix cow and buffalo milk in 1:1 ratio
↓
Adjust fat to 3%
↓ ← Add vitamin or mineral
Mix by magnetic stirrer (10 minutes)
↓
Pasteurisation (63ºC/30 minutes)
↓
Cooling (4ºC)
↓
Packaging
↓
Storage

Figure 3.1: Procedure for fortification of liquid milk

3.7 Screening and optimisation of fortificants

Screening of the fortificants and optimisation of their level of addition was done on the basis of their sensory scores, pH, acidity and TBA value.

3.7.1 Sensory evaluation

Sensory evaluation was done by a panel of ten trained judges who were asked to grade fortified milk for any change in colour and appearance, odour, taste and mouthfeel compared to control milk. Composite scoring card for sensory analysis of pasteurised milk as given by BIS (IS: 7768 1975) was used, with slight modifications. In flavour characteristics, the main focus was on metallic, rancid and oxidised flavour. Other mentioned flavour characteristics were excluded. The composite sensory score card is given as Annexure I. All sensory assessments took place at Dairy Chemistry Department of National Dairy Research Institute, Karnal, Haryana, India. The sensory booth environment was held at a constant temperature (20°C), red lighting was used to obscure any colour differences between the samples and a positive airflow removed any odours from the testing area. Saline water (0.89% sodium chloride solution) (at room temperature) was provided as palate cleanser for rinsing mouth and cleaning the tongue before testing each sample.

3.7.2 pH

pH of control and fortified milk samples was determined electrometrically with mains operated pH meter by the method described in IS: SP:18, part XI (1981). The pH meter was first calibrated using standard buffers of pH 4.0 and 9.2 and standardised using pH buffer of 7.0 at 20.0±0.1ºC.

3.7.3 Titratable acidity

Titratable acidity of control and fortified milk was determined as per IS: SP: 18, Part XI (1981).

Phenolphthalein indicator solution

1 g of phenolphthalein was weighed and taken in a 100 ml volumetric flask containing about 50 ml of 95% ethanol. The flask was stoppered and shaken vigorously for few minutes. 20 ml more ethanol was then added and shaken until a clear solution was obtained and volume was finally made to 100 ml with 95% ethanol.

Standard aqueous sodium hydroxide solution (0.1N)

0.1 N aqueous NaOH solution was prepared which was colourless and stored in an amber coloured glass bottle.

Procedure

10 ml of thoroughly mixed milk was pipetted into 150 ml conical flask. Three to four drops of phenolphthalein indicator solution (1.0%) were added to the flask. The contents of flask were titrated against standard sodium hydroxide solution (0.1 N) added drop by drop from the burette until persistent pink colour appeared and the volume of 0.1 N NaOH consumed was noted down as titre value.

3.7.4 Thiobarbituric acid (TBA) value of milk

TBA value of milk was determined by the method of King (1962). Milk samples were warmed to 30ºC and accurately 17.6 ml of milk was pipetted into a flask fitted with glass stoppers. 1 ml of TCA solution (containing 1 g TCA/ml) was added followed by 2 ml of 95% ethanol. The flask was stoppered and contents were shaken vigorously for 10 seconds. After 5 minutes, contents were filtered through Whatman filter paper no. 42. 4 ml of clear filtrate was taken in a test tube, 1 ml of TBA reagent (made by dissolving 1.4 g of 2-TBA in 100 ml 95% ethanol) was added. The contents were stoppered, mixed and placed at 60ºC in a water bath for 60 minutes. After cooling to room temperature, optical density was spectrophotometrically recorded at 532 nm. Reagent blank was also run simultaneously replacing distilled water with milk.

3.8 Iron microencapsulation

3.8.1 Preparation of iron microcapsules by spray cooling method using sodium alginate and pectin as wall material

The method of Jayalalitha *et al*. (2012) was followed to prepare iron microcapsules with certain modifications. For microcapsule preparation, 150 mg ferrous sulphate heptahydrate was dissolved in 100 ml of double distilled water and 2 g sodium alginate was added with continuous stirring. When sodium alginate+pectin were to be used as wall material, 3 g pectin was also added in the sodium alginate solution after 5 minutes of mixing and mixed thoroughly to disperse the contents uniformly. The contents were then degassed under vacuum. A droplet generator with nozzle size 1 mm was used to atomise the contents into 1 M calcium chloride solution by using nitrogen gas. To harden the microcapsules, the dispersion fluid (calcium chloride solution) along with the microcapsules was kept under refrigerated conditions (4-7ºC) undisturbed for 3-4 hrs. After this, the microcapsules thus obtained were filtered through muslin cloth, dried using freeze dryer (-50^{0}C, 50 mTorr) and stored at -20ºC.

3.8.2 Preparation of iron microcapsules by spray cooling method using polyglycerol monostearate as wall material

The method of Kwak *et al*. (2003) was followed to prepare iron microcapsules. For the microencapsulation of iron compound, polyglycerol monostearate (PGMS) was used as wall material and ferrous sulphate was selected as core material. The ratio of wall to core materials to distilled water was 5:1:30 (w/w/v). The solution of PGMS and distilled water (spray solution) was heated at 55ºC for 20 minutes and mixed thoroughly with stirring at 1200 rpm. An airless paint sprayer was used to nebulise the wall material-iron emulsion into a cylinder containing a 0.05% Tween 60 (polyethylene sorbitan monostearate) solution. The diameter of the nozzle orifice was 1.0 mm; microcapsules were formed as lipid solidified in the chilled fluid. The chilled fluid was centrifuged at 2490×g for 10 minutes to separate iron microcapsules which were then freeze dried (-50^{0}C, 50 mTorr).

3.8.3 Microencapsulation of iron by formation of liposomes

Method of Xia and Xu (2005) was followed for preparation of ferrous sulphate heptahydrate liposomes.

Preparation of citrate phosphate buffer (aqueous phase)

105 mg citric acid and 71 mg di-sodium hydrogen phosphate were dissolved in 50 ml of water and pH of buffer was adjusted to 6.8.

Procedure

For iron microencapsulation, 1.14 g lecithin (soya lecithin/egg lecithin) and 0.06 g cholesterol were dissolved in 30 ml diethyl ether (organic phase). The organic phase was then mixed with 10 ml of citrate phosphate buffer solution (aqueous phase; pH 6.8) containing 0.438 g ferrous sulphate heptahydrate and 0.03 g L-ascorbic acid. The mixture of aqueous and organic phase was placed in ice bath and sonicated using a probe sonicator at 5ºC and 5.0 second pulse rate until a stable emulsion was formed. The organic solvent was then evaporated using rotary evaporator (absolute pressure 300 mbar, temperature 60ºC) and a gel was formed. The gel was broken after persistent evaporation in the rotary evaporator. The aqueous phase (20 ml) containing Tween 80 (20% v/v) was added to the broken gel and evaporation was continued for 30 minutes. The liposomes thus formed were then dried in freeze dryer (-50ºC, 50 mTorr) and stored at -20ºC.

3.8.3.1 Microscopic observation of egg lecithin liposomes

The liposomes were diluted with distilled water (1:5). The homogeneous samples were then spread on glass slides and observed (magnification 400-fold) under an inverted light microscope and their photographs were taken by a fitted digital camera. The size of the microcapsules visible in the photographs was measured with the help of inbuilt software.

3.8.3.2 Encapsulation efficiency

Iron content of liposomes and encapsulation efficiency were determined by the method described by Abbasi and Azari (2011). For calculation of total iron in liposomes, they were ashed (at 450ºC for 4 hrs), solubilised in 5 ml of 3 M HCl and diluted in a volumetric flask with de-ionised water to 100 ml. The iron content of solutions was then measured at 248.3 nm by atomic absorption spectrophotometer.

To measure the microencapsulated iron content of liposomes, they were filled in a cellulose membrane bag (molecular weight cut off 12000 Da) and dialysed against de-ionised water for 24 hrs at 4ºC where the unencapsulated iron was removed. The liposomes which remained inside the bag after 24 hrs were then treated in the same way described for total iron in liposomes. The encapsulation efficiency was calculated by the following equation:

$$\text{Encapsulation efficiency} = \frac{\text{Total iron content of liposomes x 100}}{\text{Microencapsulated iron}}$$

3.9 Heat stability

After the adjustment of pH as described below, the heat stability of all the lots was determined as heat coagulation time (HCT) according to the method of Davis and White (1966) as modified by Jairam *et al.* (1976).

3.9.1 Adjustment of pH

Each milk sample (fortified and unfortified) was divided into eight lots of 50 ml each and adjusted to 20±0.1ºC in a water bath. The first lot was kept as control and the pH of other lots was adjusted between 6.4 to 7.0 at 0.1 pH unit interval with addition of acid or base as the case may be. After the adjustment of pH, all the lots of milk were kept at refrigerated temperature (4-7ºC) for 30 minutes and temperature was then brought to 20±0.1ºC in a water bath. The pH of the lots was readjusted with the addition of either acid or base as the case may be. Food grade stabilisers viz. sodium dihydrogen phosphate and disodium hydrogen phosphate were used for adjustment of milk pH.

3.9.2 Determination of heat stability

For the determination of heat stability of individual milk samples, 2 ml of milk from each lot was taken in corning glass tube (10 cm in length with 8 mm internal diameter) in duplicates and corked at both ends with silicon rubber corks. The tubes were then rocked at the rate of 8 cycles per minute in a metal carriage inside the hot paraffin oil (140±1.0ºC) in a thermostatically controlled bath. The heat coagulation time (HCT) in minutes was recorded as the time elapsed between the moment the tubes were dipped into oil and appearance of first visible clot. To facilitate the observation of clotting, a lamp was used which illuminated the samples from above. The heat coagulation time was recorded using a stopwatch. The graphs were then drawn between heat coagulation times (in minutes) on Y-axis and pH on X-axis. Heat stability was determined from the maxima of the HCT/pH graph thus obtained.

3.10 Determination of vitamin A

Method of Kazmi *et al.* (2007) was adopted for extraction of vitamin A from control and fortified milk. A few modifications were incorporated for breaking the wall material of the encapsulated vitamin A used for milk fortification. Vitamin A was measured and reported as IU/L. One IU of vitamin A acetate or palmitate is equivalent to 0.33 µg vitamin A.

Columns and cartridges

Analytical column : Phenomenex, Luna 5 µm C_{18}, 100Å, 250×4.6 mm, Torrance, USA
Guard column : Delta-Pack, 5 µm C_{18}, 100Å, (Waters, USA)
Column holder : Delta-Pack, Waters, Massachusetts, USA
Precolumn filters : Ultra-low dead volume with 0.5 µm frits

Preparation of reagents

Potassium hydroxide (60%): Solution was prepared fresh daily. 15 g of potassium hydroxide (KOH) was weighed and transferred into a 25 ml volumetric flask and volume was made up to the mark with distilled water.

Ammonium hydroxide (2.5%): 10 ml of 25% ammonium hydroxide was taken in 100 ml volumetric flask and volume was made up to the mark with distilled water.

HCl (0.1 N): 8.3 ml of concentrated HCl was pipetted in 1000 ml volumetric flask and volume was made up to the mark with distilled water.

Pyrogallol (30%) (ethanolic): 3 g pyrogallol was weighed in 10 ml volumetric flask and volume was made up to the mark with distilled water.

Preparation of vitamin A standard (all trans retinol)

13.2 mg retinol was accurately weighed and transferred into 10 ml low actinic volumetric flask and dissolved in HPLC grade methanol and volume was made up to 10 ml. This stock solution had vitamin A concentration of 435600 IU/L and was diluted to such an extent that the resulting solutions were having concentration of 100, 1000, 2000, 3000 and 4000 IU/L. The standard curve of vitamin A is given below in figure 3.2. The standard solutions were stored in a low actinic flask (amber) which remained stable for at least one and four weeks at 4 and -20ºC, respectively. Figure 3.3 depicts the chromatogram showing peak of vitamin A extracted from vitamin A fortified milk by the standardised method. Recovery of standardised method was 95-99.8%.

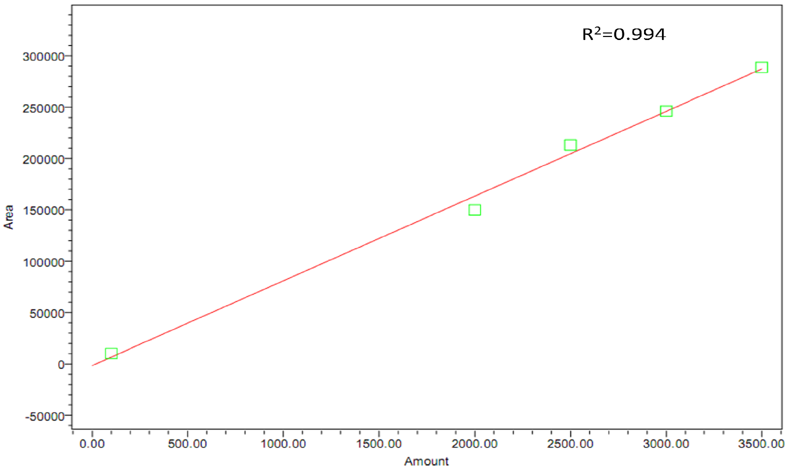

Figure 3.2: Standard curve for estimation of retinol content

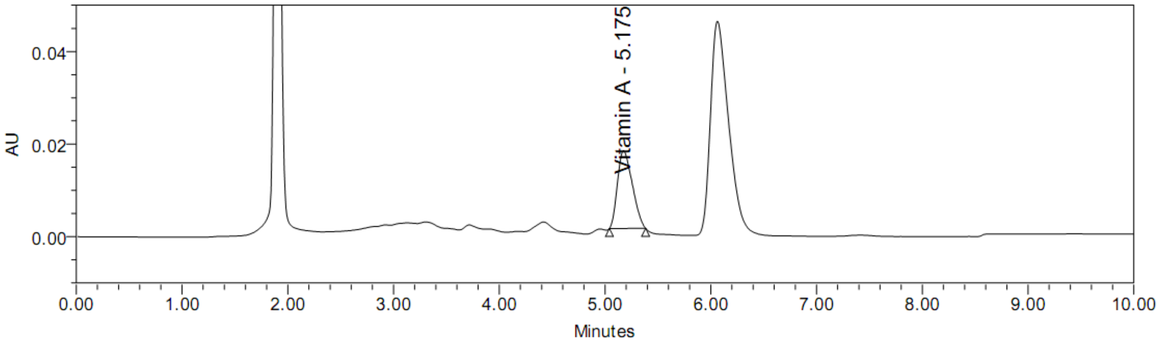

Figure 3.3: Chromatogram of vitamin A extracted from milk

3.10.1 Sample preparation

Milk samples were pasteurised at 63ºC for 30 minutes in high precision water bath in air tight glass bottles. The samples were immediately cooled to 4ºC. After 2 hrs of storage at 4ºC, milk samples were analysed for vitamin A content.

3.10.2 Extraction of vitamin A from milk

2 ml milk was taken in centrifuge tube. 1 ml of ammonium hydroxide solution was added to it, the contents were sonicated for 10 minutes and then vortexed. 1 ml 0.1 M hydrochloric acid was added, vortexed and sonicated for 10 minute again. 1 ml of aqueous potassium hydroxide (60%, w/v) and 100 µl of alcoholic pyrogallol (30%, w/v) were added, the contents were flushed with ultrapure nitrogen gas and then kept for saponification at 70ºC for 30 minutes in water bath. The contents were then cooled to room temperature in an ice bath and vortexed. 2 ml hexane was added, contents were vortexed and a few drops of ethanol were added and then centrifuged at 1800 rpm for 10 minutes. Upper hexane layer was transferred to another centrifuge tube with the help of a pipette. This extraction step was repeated twice by adding 2 ml hexane each time and all the extracts were collected in centrifuge tubes. The collected extracts were dried under nitrogen. The dried material was reconstituted to 1 ml with mobile phase, transferred to a 2 ml vial and centrifuged in refrigerated centrifuge at 4000 rcf for 15 minutes at 0ºC. The supernatant was then filtered through 0.22 µm syringe filter and 20 µl of filtrate was then injected in HPLC system.

3.10.3 Chromatographic conditions

Reverse phase HPLC analysis procedure was standardised for vitamin A, selecting acetonitrile:chloroform:methanol (88:4:8) as mobile phase, with flow rate 1ml/minute, in C_{18} column, temperature 40^{o}C and PDA detector (λ^{max} 325 nm).

3.11 Analysis of iron content

Iron was analysed using atomic absorption spectrophotometer (AAS) by AOAC (2005) using dry digestion method.

Cleaning of glass ware

All glassware used for AAS analysis were washed properly and soaked in potassium dichromate solution, then washed two times with single distilled water and then with double distilled water.

Preparation of standard curve for iron estimation

4.98 mg ferrous sulphate heptahydrate (99% pure) was accurately weighed and transferred to 100 ml volumetric flask to prepare a stock solution of iron. 5 ml of concentrated nitric acid was added to it to dissolve properly and volume was made up to the mark with double distilled water. This stock solution (100 ppm iron) was diluted to prepare standards of desired concentration: 0.25, 0.5, 1.0, 1.5 and 2.0 ppm. The samples were analysed using atomic absorption spectrophotometer and standard curve was prepared (figure 3.4).

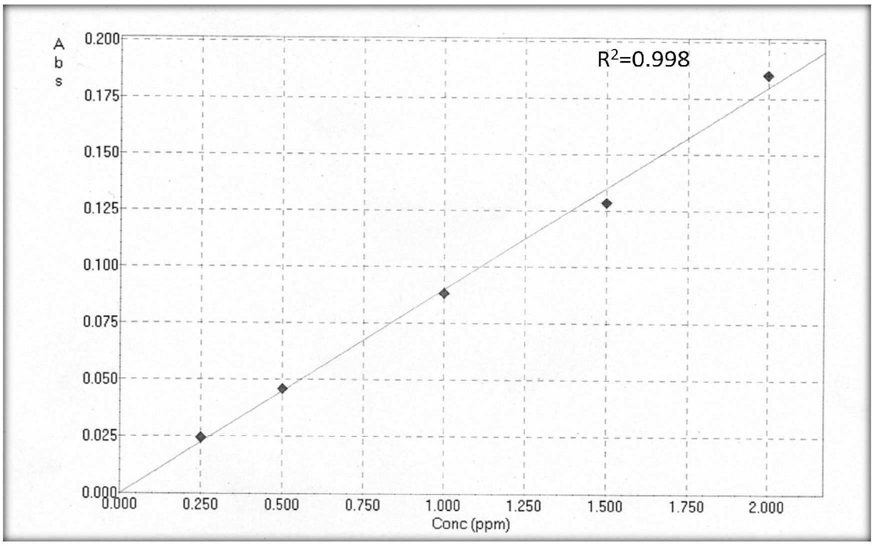

Figure 3.4: Standard curve for estimation of iron content

Procedure

5 g milk sample was taken in a silica crucible and dried on hot plate at 100ºC. The samples were then preashed on hot plate at about 250ºC. Crucible was placed in muffle furnace at 200-250ºC and temperature was raised slowly to 450ºC at a rate of not more than 50ºC/hr and kept in muffle furnace overnight. Crucible was then taken out of furnace and 1-3 ml of double distilled water was added and evaporated on hot plate. Crucible was again placed in muffle furnace at 200ºC and temperature was slowly raised to 450ºC. Ashing was continued for 1-2 hrs or until the product was completely ashed i.e. white/grey ash was obtained. Thereafter, 5 ml 6 M HCl was added to crucible ensuring that all ash comes in contact with acid. Acid was evaporated on hot plate and residue was dissolved in 0.1 M HNO_3 and the volume was made to 50 ml with the same. Iron analysis was then carried out in atomic absorption spectrophotometer (λ^{max} 283.3 nm).

3.12 Evaluation of vitamin A and iron stability during different processing and storage conditions

3.12.1 Evaluation of stability of vitamin A and iron in milk as affected by heat treatments

1. Pasteurisation (63ºC/30 minutes)
2. Boiling
3. Sterilisation (121ºC/15 minutes/15 psi)

Milk samples were analysed for vitamin A and iron using HPLC and AAS, respectively to detect their stability in milk as affected by different heat treatments.

3.12.2 Evaluation of stability of vitamin A and iron in milk as affected by exposure to light of different intensities

The control and fortified milk samples were pasteurised at 63ºC for 30 minutes. The samples were cooled and kept in glass test tubes covered with parafilms and stored at 4ºC temperature in BOD incubator so that maximum portion of milk was exposed to light. Three different light intensities were used viz. 1485, 2970 and 4455 lux for 32 hrs at 4ºC. Milk samples were analysed at fixed intervals

(0, 2, 4, 8, 16 and 32 hrs) for vitamin A and iron using HPLC and AAS, respectively to determine their stability as affected by light intensity and duration of exposure.

3.12.3 Evaluation of stability of vitamin A and iron in milk as affected by storage (4-7ºC) in different packaging materials

Control and fortified milk samples were pasteurised at 63ºC for 30 minutes. The samples were cooled, packed in two different packaging materials viz. glass bottles and polyethylene pouches and stored under refrigerated conditions (4-7ºC). Milk samples were analysed at fixed intervals (0, 3rd, 5th and 7th day) for vitamin A and iron using HPLC and AAS, respectively to detect their stability in milk as affected by storage in different packaging materials.

3.13 Physico-chemical properties

3.13.1 Oxidative stability

Control and fortified milk samples were pasteurised and stored in polyethyelne pouches and glass bottles under refrigerated conditions (4-7ºC) for seven days. Cream was separated from each sample on 0, 3rd, 5th and 7th day of storage and clarified into ghee at 120ºC by direct creamery method. These ghee samples were used to determine the oxidative stability of control and fortified milk by determination of following parameters:

3.13.1.1 Peroxide value

Peroxide value of ghee samples was determined by the method as described in IS: 3508 (1966). The details are given below:

Preparation and standardisation of 0.1 N $Na_2S_2O_3$ solution (stock solution)

Approximately 25 g of sodium thiosulphate ($Na_2S_2O_3$) crystals were dissolved in well boiled and CO_2 free water and volume was made up to 1000 ml. The solution was stored in a dark coloured reagent bottle and standardised as follows:

5 g of finely ground potassium dichromate ($K_2Cr_2O_7$) was accurately weighed and transferred to a clean volumetric flask (1000 ml). Some distilled water was poured into the volumetric flask and it was thoroughly shaken and finally volume was made up to the mark. The solution was kept in a cool, dry place. 25 ml of this solution was pipetted into a clean glass stoppered 250 ml conical flask. 5 ml of

concentrated HCl and 15 ml of 10% potassium iodide solution were added. The solution was then allowed to stand in the dark for 5 minutes. and the mixture was titrated against $Na_2S_2O_3$ solution (0.1 N as prepared above) using 2 ml of starch solution as an indicator towards the end. The end point was the change in blue colour to green colour.

The normality of $Na_2S_2O_3$ solution was calculated as follows:

$$\text{Normality of } Na_2S_2O_3 \text{ solution} = \frac{25XW}{49.03XV}$$

where, W = weight of $K_2Cr_2O_7$ taken in g

V = volume of $Na_2S_2O_3$ required for titration in ml

Preparation of 0.002 N solution of $\mathbf{Na_2S_2O_3}$

Prepared freshly by diluting the previously prepared 0.1 N $Na_2S_2O_3$ solution

Saturated potassium iodide (KI) solution

Some quantity of water was boiled for 5 minutes and allowed to cool. To a portion, enough potassium iodide to ensure a saturated solution (10 g KI in 6 ml water) was added.

Starch indicator (1%)

1 g of soluble starch was weighed and paste was made in 30 ml of water. The paste was transferred to 80 ml boiling water and heated until a clear solution was obtained. The contents were cooled and stored in a tight stoppered bottle.

Mixed solvent

Glacial acetic acid:chloroform, 2:1 (v/v)

Procedure

1 g of molten fat was weighed accurately into a 250 ml glass stoppered Erlenmeyer flask. 20 ml of mixed solvent was added and flask was then swirled until the dissolution of fat. 0.5 ml of saturated KI was then added to the flask and kept undisturbed for 1 minute. The mixture was heated on boiling water bath up to boiling and tip of the flask was closed with finger after vapours started to form and boiling

was continued for 30 seconds. Generated vapours were condensed under stream of running tap water. 25 ml of distilled water was added followed by 0.5 ml of 1% starch indicator. At this point dark blue/brown colour appeared. The mixture was then titrated against 0.002 N $Na_2S_2O_3$ until disappearance of colour. Blank without sample was also run simultaneously.

Calculation

The peroxide value as milliequivalents of peroxide oxygen per kg of fat was calculated as:

$$\text{Peroxide value} = \frac{(S-B) \times N \times 1000 \times 8}{W}$$

where, S = volume of $Na_2S_2O_3$ required in titration of sample in ml

B = volume of $Na_2S_2O_3$ required in titration of blank in ml

N = exact normality of $Na_2S_2O_3$ solution

W = weight of sample in g

Note: The peroxide value as millimoles of oxygen per kg of fat was calculated as: peroxide value/16

3.13.1.2 Free fatty acid content

Free fatty acid (FFA) content of ghee was determined by the method as described in IS: 3508 (1966). The details of the method are given below:

Neutralised ethanol

99% ethanol was titrated against standardised alkali in the presence of phenolphthalein indicator until a faint pink colour was obtained that persisted for 15-30 seconds.

Procedure

5.0 g of fat sample was accurately weighed in a 250 ml conical flask. In a second flask, 50 ml of ethanol was heated to boiling point and while still above 70ºC, it was neutralised with 0.1 N sodium hydroxide solution using phenolphthalein as an indicator. The neutralised alcohol was poured into the flask containing ghee. The contents were brought to boiling temperature on a boiling water bath. The solution, while hot, was titrated against 0.1 N sodium hydroxide solution, shaking vigorously during titration. The end point of the titration was perceived when the addition of a single drop produced a slight but definite colour change which persisted for at least 15 seconds. The FFA content was expressed as percent oleic acid.

Calculation

$$\text{FFA (\% oleic acid)} = \frac{2.82 \times V}{W}$$

where, V = volume of 0.1 N sodium hydroxide used in ml

W= weight of the sample in grams

3.13.1.3 Thiobarbituric acid (TBA) value

TBA value of ghee samples was determined by the method as described by Patton and Kurtz (1951). The details of the method are given as follows.

TBA reagent

0.76 gm of 2-thiobarbituric acid (TBA) was weighed accurately and transferred to a 100 ml volumetric flask. Some distilled water was added and TBA was dissolved by heating on a water bath, volume was made up to the mark with distilled water and solution was then filtered. Filtrate was mixed with glacial acetic acid in a ratio of 1:1.

Procedure

3.0 gm of molten ghee sample was accurately weighed into a glass stoppered test tube and to this 10 ml of carbon tetrachloride solvent and 10 ml of TBA reagent were added. The contents were shaken vigorously i.e. about 125 oscillations/minute for 4 minutes. The test tubes were left undisturbed to obtain two clearly separated layers. 5.0 ml portion of the separated upper layer was transferred to another test tube using a pipette. The contents were then incubated in a boiling water bath for 30 minutes. Blank sample was also prepared simultaneously by replacing 3.0 gm sample with 3.0 ml carbon tetrachloride. Readings were taken as optical density (OD) at 532 nm using a spectrophotometer.

3.13.1.4 Induction period (oil stability index)

The Rancimat apparatus was used to measure the induction period of ghee samples prepared from control and fortified milk samples to evaluate the effect of vitamin A and iron addition on oxidative stability of milk fat under accelerated oxidative conditions.

Procedure

Prior to the use of the Rancimat, all glasswares were thoroughly cleaned with double distilled water and alcohol. A 3.0 g sample of completely melted ghee was weighed accurately into each of the reaction vessels. The vessels were then placed in the heating block of the Rancimat apparatus. The reaction vessels were then connected to the measuring vessels via connecting tube. 60 ml of deionised water was measured into each of the measuring vessels, containing the electrodes. The measuring vessels were also placed in the Rancimat apparatus. All parts were connected to the apparatus as per the operating instructions and the test was carried out until the endpoints of all the samples were reached, with a maximum allowable limit of 48 hrs. Temperature of heating blocks was set at 120ºC and a constant air flow rate (20 L/hr) was maintained throughout the run.

3.13.2 Curd tension

Curd tension of milk was determined as per the method described by Chandrasekhara *et al*. (1957). A simple type of curd tension meter was employed. It consisted of three sharp stainless steel knives of the size 1”×1/4”, welded in form of “H”. A thin vertical rod was bent in the form of a hook which was attached to the thread carrying the pan over a frictionless pully. The curd tension of milk samples was determined in uniform size beakers of 100 ml capacity. 50 ml milk was taken in a beaker and pre-heated at 30±1ºC. The curd tension knife was then placed in the beaker and 0.5 ml of 0.015% (w/v) Meito rennet was added. Milk was stirred immediately and was placed in an incubator maintained at 30±1ºC. The pan was loaded with weights, till the curd tension knife was able to cut its way through the curd. The weight expressed in grams was taken as a measure of curd tension.

3.13.3 Absolute viscosity

Kinematic viscosity of samples at 27ºC was measured with an Ostwald's U-tube viscometer. The density of samples at 27ºC was analysed using pycnometer (Lewis 1996). The experiment was conducted using thermostatically controlled transparent glass water bath to maintain temperature exactly at 27ºC. Absolute viscosity was calculated according to equation:

$$\text{Absolute viscosity} = \text{Kinematic viscosity} \times \text{density}$$

3.13.4 Rennet coagulation time (RCT)

Rennet coagulation time (RCT) of control and fortified milk was determined according to the method described by Berridge (1952).

Rennet solution: 300 mg of microbial rennet was dissolved in 100 ml of distilled water.

Procedure

10 ml milk was taken in a test tube and 1 ml of 0.2% rennet solution was added to it. The contents were mixed by inverting the test tube at an angle of 45ºC and observed for first clot at regular intervals. The time taken from point of addition of rennet solution to milk, till observation of the first clot was noted as rennet coagulation time. The experiment was conducted using thermostatically controlled water bath at 30±1ºC.

3.13.5 Alcohol stability

Alcohol stability of control and fortified milk was determined as per IS: SP: Part XI (1981).

Procedure

5 ml of milk was pipetted in a test tube and an equal quantity of alcohol (68% by weight or 75% by volume; density 0.8675 g/ml at 27ºC) was added to it. The contents were mixed by inverting the test tubes several times. Samples were observed for presence of any flakes or clots. The presence of a flake or clot denotes a positive test.

3.13.6 Dry sediment (%)

An accurately weighed sample (approximately 2 g) was added to a centrifuge tube. The tube was then centrifuged at 15000 rpm for 30 minutes (2760 rcf) in a centrifuge. The supernatant was discarded and the pellet was transferred to a moisture dish and dried in a hot air oven at 105ºC to a constant weight. The equation below was applied to find % dry sediment:

$$\% \text{ Dry sediment} = B/A*100$$

where, A= weight of sample in grams; B= weight of dried pellet in grams

3.13.7 Colour estimation

Hunter colorimeter was used to measure the degree of change in colour produced by addition of iron salts to milk. The colour coordinates of this meter were L=whiteness; a=redness to greenness and b=yellowness to blueness. The instrument was standardised with standard reference tile, coordinates for the tile were L=50.83 to 93.00; a= 0.92 to -26.27 and b= 1.70 to 12.12. Milk samples were pasteurised (63ºC/30 minutes) and stored under refrigerated conditions (4-7ºC). After 2 hrs, milk samples were brought to room temperature and colour measurements were performed.

3.14 Distribution of iron in different milk fractions

The method described by Unnikrishnan and Rao (1977a) was used with slight modifications. Milk was fortified with selected iron salts at a level of 25 ppm iron and pasteurised at 63ºC for 30 minutes. Glasswares were repeatedly washed with double distilled water to avoid iron contamination. To follow the migration between cream and skim milk, samples were centrifuged at 4000 rpm for 30 minutes at 20ºC. The centrifuged samples were hardened in ice bath and cream layers were punctured with the help of a pointed glass rod and the skim milk was drawn off. The cream so obtained was clarified into ghee and separated from ghee residue by filtration. Casein was prepared from skim milk by isoelectric precipitation at pH 4.6 with 1 N HCl. The casein thus obtained was washed repeatedly with double distilled water and washings were added to whey. Estimation of iron in milk fractions was done using atomic absorption spectrophotometer (AAS).

3.15 Fractionation of iron in colloidal and soluble phase (ultracentrifugation)

Control and fortified milk samples were separated into colloidal and soluble phases by centrifugation at 110000 rcf for 60 minutes at 20ºC (30700 rpm) using an ultracentriguge. Under these conditions, the composition of the supernatant phase can be viewed as an empirical guide to the composition of the serum phase of the original milk. Following centrifugation, supernatants were discarded, pellets were collected and analysed for iron content using atomic absorption spectrophotometer.

3.16 Effect of vitamin A and iron on electrophoretic behaviour of milk proteins

The effect of iron and vitamin A fortification on electrophoretic mobility of milk proteins was determined using urea-PAGE and SDS-PAGE.

3.16.1 Urea-polyacrylamide gel electrophoresis (urea-PAGE) of milk proteins

Alkaline urea-PAGE resolves casein well. A high concentration of urea is incorporated into both the gel systems and sample buffer. The chaotropic nature of urea disrupts the association between casein species, allowing proteins to migrate as monomer. Method described by Simon (2004) was used for urea-PAGE.

Reagents for urea-PAGE

1. Stock acrylamide solution: 30% (w/v) acrylamide, N, N'-methylene-bis-acrylamide (BIS) (37.5:1), electrophoresis grade
2. 15% (w/v) ammonium persulphate (APS) in double distilled water
3. N, N, N', N'-Tetramethylenediamine (TEMED)
4. 0.4% (w/v) bromophenol blue in double distilled water
5. Resolving buffer: 0.38 M Tris-HCl, pH 8.8 (adjusted with 6 M HCl), 4.5 M urea
6. Stacking gel buffer: 90 mM Tris-HCl, pH 8.4 (adjusted with 6 M HCl), 0.6 M urea, 90 mM boric acid, 2.5 mM EDTA
7. Sample buffer: 90 ml alkaline urea stacking gel buffer, 2 ml 0.8% bromophenol blue, 8 ml glycerol
8. Electrode buffer: 17.6 mM Tris-HCl, pH 8.4 (adjusted with 6 M HCl), 17.8 mM boric acid, 0.55 mM EDTA
9. Staining solution: 0.05% Commassie Brilliant Blue R 250 in isopropanol/acetic acid/water (2.5:1:6.5, by volume)

10. Destaining solution: Isopropanol/acetic acid/water (1:1:8, by volume)
11. **Sample preparation**

    Control and fortified milk samples were skimmed and diluted 10 times with sample buffer. The diluted samples were heated for 4 minutes in boiling water bath and then cooled to room temperature.
12. **Preparation of casein**

    Casein was prepared by precipitation of skim milk by adjusting the pH to 4.6 with 1 N hydrochloric acid. The precipitated casein was washed four times with water and resuspended in phosphate buffer (pH 6.9). Casein was used as standard to identify major caseins and injected in same quantity after treating with sample buffer.

Resolving Gel

Gel system		**Quantity**
Acrylamide stock	:	4.00 ml
Resolving buffer	:	5.95 ml
Water	:	3.75 ml
Ammonium persulphate	:	100 µl
TEMED	:	5 µl

Stacking gel

Gel system		**Quantity**
Acrylamide stock	:	1.30 ml
Stacking buffer	:	8.65 ml
Ammonium persulphate	:	100 µl
TEMED	:	10 µl

The working solutions were filtered and stored at 4-7°C. Solutions were brought to room temperature before use.

Procedure

The glass plates were cleaned thoroughly with distilled water and ethyl alcohol. Gaskets were cleaned and plates were fixed together with help of plastic gaskets and clamps. The required quantity of the gel ingredients was measured except APS and TEMED into a 100 ml Buchner flask and contents were degassed under vacuum, with stirring for 15 minutes. APS and TEMED were added gently, swirling after each addition. 3.30 ml of the resolving gel solution was poured in between the assembled gel equipment. The gel was overlaid carefully and gently with 200 µl of water, pipetting down the sides of the glass plates. The gel was allowed to polymerise at room temperature for at least 30 minutes before draining off the water. Stacking gel was then prepared in the same manner by adding the required quantity of ingredients mentioned in the composition of stacking gel. Well forming clamps were inserted into the stacking gel solution immediately after pouring the stacking gel solution in between the plates avoiding trapping of air bubbles underneath the combs. The gel was allowed to polymerise at room temperature for at least 1 hr. The combs were removed and sample wells were overlaid with enough water to fill completely. Clamps and combs were removed from the plates. The electrode buffer was poured in the container upto ½ level and then glass assembly was placed inside avoiding formation of air bubbles. The container was then filled with the electrode buffer upto the level of plates filling the entire well completely without overflow. 10 µl of diluted sample was injected into the well with the help of gel loading tips and the electrodes were connected properly. Gel was run according to appropriate electrophoresis conditions given below:

Voltage	:	210 Volts
Current	:	70 mA
Power	:	6.5 W
Time	:	0.9/1.1 hrs

Gel staining and destaining

The unit was dismantled after the completion of run and plates were detached carefully avoiding the breakage of gel. The gel was transferred to the staining solution and kept for 1 hr after which the gel was transferred to destaining solution. Destaining was done overnight, till the bands appear and background become clear. The gel was then transferred to distilled water and photographs of the gel were taken with the help of a light source. The intensity (height and raw volume) of bands was measured with the help of densitometer.

3.16.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of milk proteins

SDS is a negatively charged surfactant. It is used to disrupt non covalent bonds through its ability to absorb to hydrophobic and positively charged sites on proteins. Most proteins bind similar amounts of SDS on a mass basis. The SDS-protein complexes may then be separated on the basis of molecular size of the protein. Samples are normally reduced by treatment with 2-mercaptoethanol to disrupt intra- and intermolecular disulfide bonds. SDS-PAGE was performed according to the method described by Simon (2004).

Reagents for SDS-PAGE

1. Stock acrylamide solution: 30% (w/v) acrylamide, N, N'-methylene-bis-acrylamide (BIS) (37.5:1), electrophoresis grade
2. 15% (w/v) ammonium persulphate (APS) in double distilled water
3. N, N, N', N'-Tetramethylenediamine (TEMED)
4. 10% (w/v) SDS in double distilled water
5. 0.4% (w/v) bromophenol blue in double distilled water
6. Resolving gel buffer: 1.5 M Tris-HCl, pH 8.8 (adjusted with 6 M HCl)
7. Stacking gel buffer: 0.5 M Tris-HCl, pH 6.8 (adjusted with 6 M HCl)
8. Sample buffer: 12.5 ml SDS stacking gel buffer, 50 ml water, 2.5 ml 0.4% bromophenol blue, 10 ml glycerol and 20 ml 10% SDS were mixed together to prepare sample buffer
9. Electrode buffer: 25 mM Tris-HCl, pH 8.6 (adjusted with 6 M HCl), 190 mM glycine
10. Staining solution: 0.05% Commassie Brilliant Blue R 250 in isopropanol/acetic acid/water (2.5:1:6.5, by volume)
11. Destaining solution: Isopropanol/acetic acid/water (1:1:8, by volume)
12. **Sample preparation**

    Control and fortified milk samples were skimmed and diluted 10 times with SDS sample buffer containing 20 µl mercaptoethanol/ml. The diluted samples were heated for 4 minutes in boiling water bath and then cooled to room temperature.

Resolving Gel

Gel system		Quantity
Acrylamide stock	:	4.00 ml
Resolving buffer	:	2.25 ml
Water	:	2.00 ml
10% SDS stock	:	100 µl
Ammonium persulphate	:	50 µl
TEMED	:	5 µl

Stacking gel

Gel system		Quantity
Acrylamide stock	:	0.65 ml
Stacking buffer	:	1.25 ml
Water	:	3.05 ml
10% SDS stock	:	50 µl
Ammonium persulphate	:	25 µl
TEMED	:	5 µl

The working solutions were filtered and stored at 6°C. Solutions were brought to room temperature before use.

Procedure

Same procedure was followed for electrophoresis run, staining and destaining as in case of urea-PAGE. Molecular weight, height and raw volume of the bands were calculated by densitometer using standard protein molecular weight markers.

3.17 Effect of iron and vitamin A fortification on Maillard browning in milk

3.17.1 Determination of 5-hydroxymethylfurfural content (HMF) content in milk

HMF content was determined following the spectrophotometric method described by Keeney and Bassette (1958). Stock solution of HMF standard (100 µM) was prepared by dissolving 12.6 mg HMF in HPLC grade water and making up the volume to 100 ml. This stock solution was further diluted to prepare standards of 0.5, 5, 10, 20, 30, 40 and 50 µM HMF concentration. To measure total HMF (free plus potential HMF) from browning intermediates, 10 ml milk was taken in a test tube, 5 ml of oxalic acid (0.3 N) was added to it and the tube was covered with an inverted beaker and placed in boiling water bath for 1 hr, after which it was cooled. 5 ml of 40% trichloroacetic acid was then added and the mixture was filtered through Whatman paper no. 42. An aliquot of filtrate (4 ml) was transferred to a test tube and 1 ml of 0.05 M 2-thiobarbituric acid (TBA) was added to it. The contents were heated at 40ºC for 30-40 minutes in a water bath and cooled to room temperature. A blank run was also conducted simultaneously substituting water for milk. Same procedure was followed for the standard solutions as in case of milk samples. Optical density was measured at 443 nm and a standard curve was prepared (figure 3.5). HMF content in milk samples was interpolated from the standard curve and results were expressed as micromoles HMF.

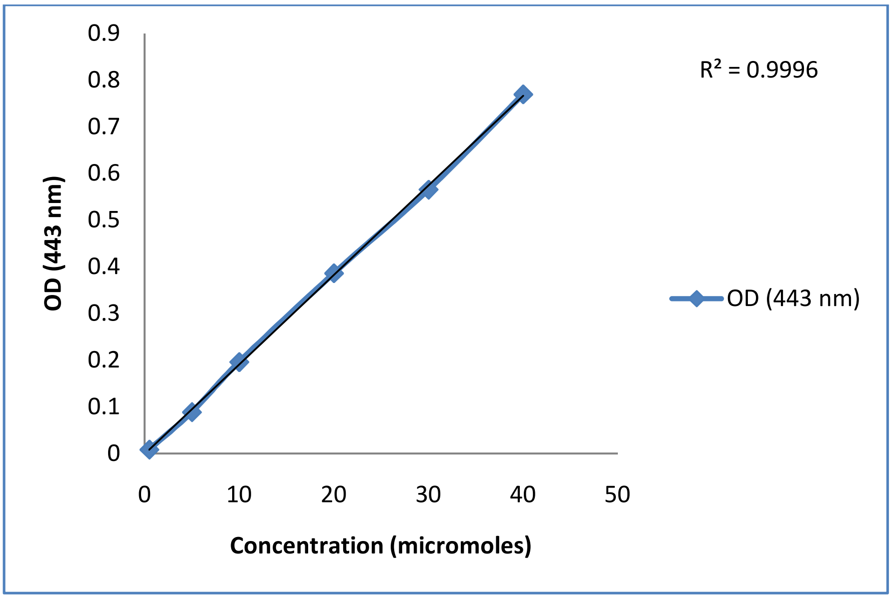

Figure 3.5: Standard curve for estimation of HMF content

3.17.2 Determination of furosine content in milk

Furosine was estimated by the method described by Birlouez-Aragon *et al.* (1997). Milk was centrifuged at 3100 rpm for 30 minutes at 10ºC to separate the cream. Milk protein was isolated from milk by vigorously mixing 5 ml milk with 0.25 ml cold NaCl (9 g/L), 2 ml absolute alcohol (containing 1 g BHT/L) and 4 ml dichloromethane. Mixture was centrifuged at 2000 rcf for 5 minutes at 4ºC to remove remaining lipid fraction from protein. The contents were then filtered and the precipitates were dried and taken in a 15 ml glass vial. Precipitates were hydrolysed by adding 8 ml of 7.8 N HCl and flushed with nitrogen and kept for heating in muffle furnace at 100ºC for 18 hrs. The dried residue was then reconstituted with 0.6 ml HPLC water and filtered through 0.45 µm membrane. Stock solution of furosine hydrochloride standard was prepared by dissolving 10 mg furosine hydrochloride in HPLC grade water and making up the volume to 10 ml. This stock solution was further diluted to prepare standards of 1, 10 20, 30, 40 and 50 mg furosine per ml. A standard curve was prepared (figure 3.6) and furosine content in milk samples was interpolated from this; results were expressed as mg furosine per g of protein.

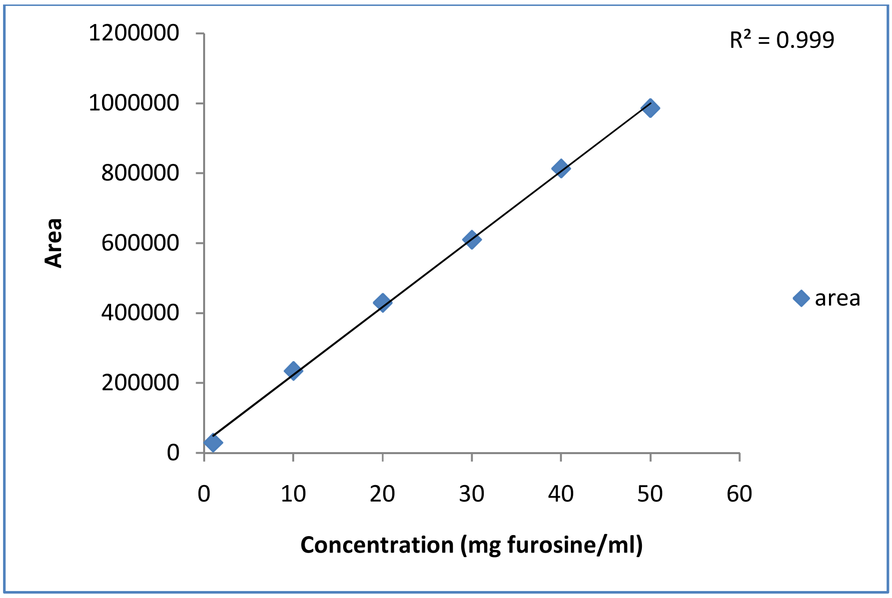

Figure 3.6: Standard curve for estimation of furosine content

HPLC conditions

Analytical column : Phenomenex, Luna 5 µm C_{18} 100Å, 250×4.6 mm, Torrance, USA

Guard column : Delta-Pack, 5 µm C_{18}, 100Å (Waters, Massachusetts, USA)

Detector : Photodiode array detector (λ^{max} = 280 nm)

Mobile phase : 5.6 mM *0*-phosphoric acid

Flow rate : 1 ml/minute

3.18 Effect of fat content (whole milk, toned milk, double toned and skim milk) on quality characteristics of milk

Control and fortified milk samples were prepared from whole milk, toned milk, double toned milk and skim milk and analysed for the effect of fat content on sensory acceptability, curd tension, viscosity and oxidative stability. These parameters were determined according to the methods described earlier.

3.19 Effect of fortification on sensory, physical and microbiological properties of *dahi*

3.19.1 Preparation of *dahi* from fortified milk

Dahi was prepared from control and fortified toned milk (cow milk: buffalo milk 1:1). Milk was warmed at temperature of 90ºC for 15 minutes and cooled to 30ºC. NCDC 167 culture (*L. lactis ssp. lactis*, *L. lactis ssp. diacetyllactis*, *L. lactis ssp. cremoris, Leuconostoc spp.*) was added at the rate of 1.5% to control as well as fortified milk, mixed thoroughly and incubated at 30ºC.

3.19.2 Sensory analysis

Sensory evaluation of *dahi* was evaluated on composite sensory score card. Parameters were colour and appearance, flavour, consistency, taste and mouthfeel and overall acceptability. A ten membered panel of judges was constituted for the sensory evaluation. The composite sensory score card is given as Annexure II. Sensory analysis was carried out as discussed in section 3.7.1 for milk.

3.19.3 Setting time

The setting time of sample (in hrs) was recorded from the time of inoculation to the time when coagulum was just formed.

3.19.4 Titratable acidity

Titratable acidity of the *dahi* samples was estimated according to the method of standard AOAC procedure (AOAC 2005). Approximately 20 g *dahi* sample was weighed in a conical flask and diluted with twice its volume with double distilled water. 2 ml of phenolphthalein indicator was added and the contents were titrated with 0.1 N NaOH to persistent pink colour. Acidity was reported as % lactic acid by weight.

Acidity (% lactic acid) = Volume of NaOH consumed

1 ml 0.1 N NaOH = 0.009 g lactic acid

3.19.5 Spontaneous syneresis in undisturbed set *dahi*

Spontaneous syneresis of undisturbed set *dahi* was determined using a siphon method used by Amatayakul *et al.* (2006). In this study, a cup of set *dahi* was taken from the set *dahi* kept in refrigerated conditions for 2 hrs, weighed and kept at an angle of approximately 45º to allow the whey on the surface to collect on the side of the cup. A needle connected to a syringe was used to siphon the liquid whey from the surface of the sample and the cup of *dahi* was then re-weighed. The siphon was carried out within 10 seconds to prevent further leakage of whey from the curd. The syneresis was expressed as the percentage weight of the whey over the initial weight of the yoghurt sample.

3.19.6 Water holding capacity

Water holding capacity (WHC) of *dahi* samples (A) was analysed by the method described by Remeuf *et al*. (2003). For this, 20 g of *dahi* sample was taken in a graduated centrifuge tube and the contents were centrifuged for 20 minutes at 669 rcf and 20ºC. The whey expelled (B) was removed and weighed. The WHC was expressed as:

WHC (%) = 100 x A-B/B

3.19.7 Viscosity (Rotational viscometer)

Viscosity of all *dahi* samples (at 20ºC) was determined using rotational viscometer which was an open, concentric measurement system and allowed measurement by immersion. The measuring head and measuring tube were rigidly coupled; the measuring unit was driven by a DC motor. Viscometer fitted with spindle TL-7 and adjusted to 10, 20 and 30 rpm. A built-in microprocessor calculated the values for the viscosity (in centipoise) with the aid of the measured torque, the set shear rate (0.28xrpm) and the measurement system used.

3.19.8 Texture profile analysis

The firmness of *dahi* prepared from control and fortified toned milk (cow milk:buffalo milk 1:1) was determined using texture analyser fitted with 5 kg load cell. For texture analysis, *dahi* samples were prepared from 80 ml milk in 100 ml glass beakers, the set samples were kept in immersion chamber maintained at 25ºC before the analysis. The samples were subjected to mono-axial compression of 20 mm distance on the texture analyser by the crosshead speed of 2 mm/second. The test conditions maintained were as under:

Measured force in compression
Probe P25 cylindrical (diameter 25 mm)
Distance = 20 mm
Load cell = 25 kg
Pre-test speed = 2 mm/second
Test speed = 2 mm/second
Post-test speed = 2mm/second
Single TPA (texture profile analysis) i.e. single time penetration

After the completion of analysis, a graph was obtained with force experienced by probe on Y-axis and time on X-axis. The firmness of *dahi* sample was estimated as the height of positive peak force upto rupture point. A representative graph obtained for milk samples is shown in figure 3.7.

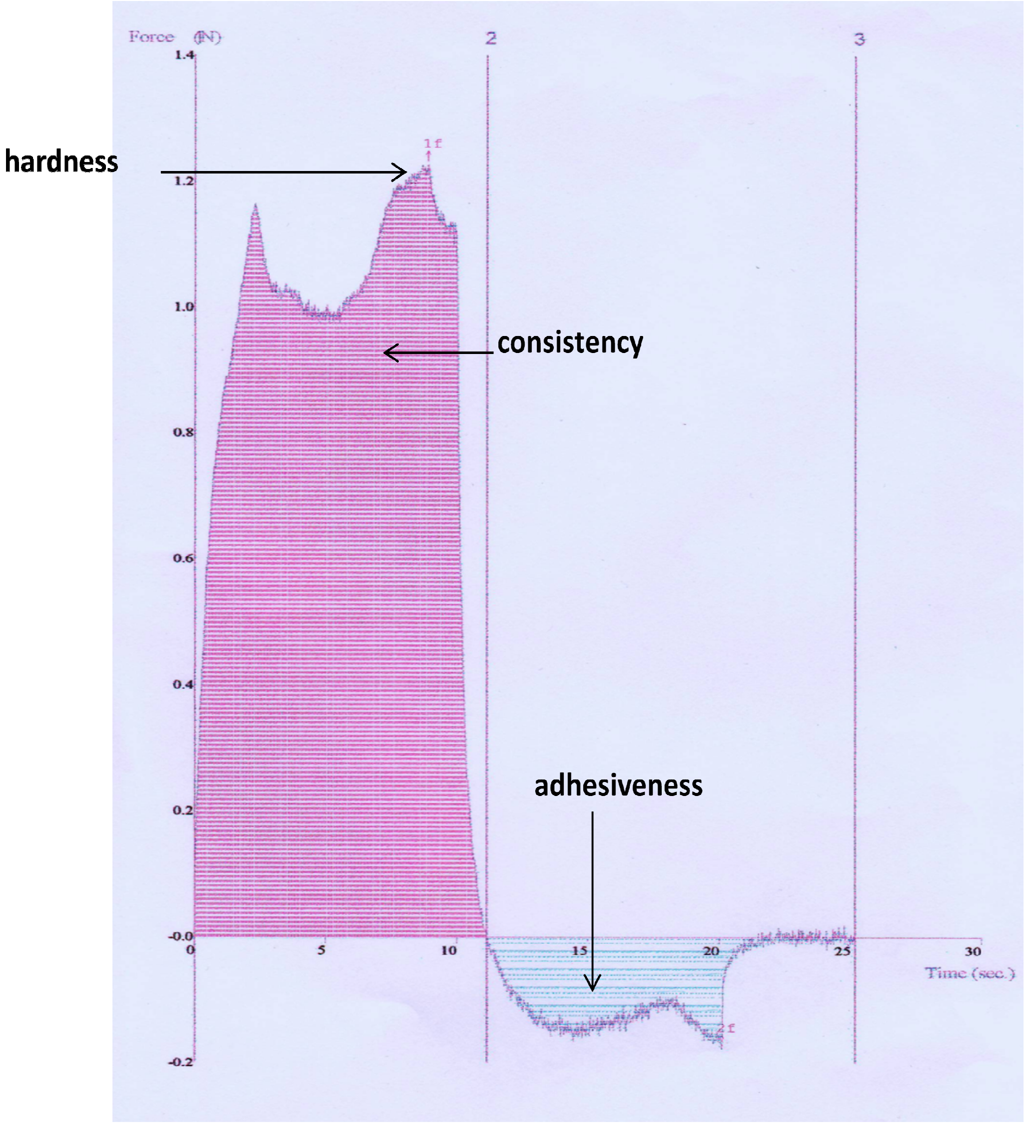

Figure 3.7: Graphic representation of measurements using texture analyser TA.XT2i

Consistency (N s) – the area of the resistance peak; maximal peak force- hardness (N)– the maximum resistance to the penetration peak (height of peak 2); adhesiveness (N s)– the area of the adhesion peak.

3.19.9 Lactococcal count

Preparation of dilution blanks

The dilution blanks consisted of 0.85% sodium chloride in 9 ml portions in test tubes. These were cotton plugged and sterilised at 121ºC and 15 psi for 15 minutes. 1 ml of sample was taken and mixed with 9 ml of dilution blank (0.85 % NaCl) to get the first dilution. It was vortexed and 1 ml of first dilution was taken and mixed with another 9 ml dilution blank to get the second dilution. Likewise up to seventh dilution was prepared.

Pour plating

Seventh dilution was taken for plating. Lactococcal count was enumerated using M17 agar. Plating of serially diluted control and fortified milk samples was done and plates were incubated at 30ºC for 24-48 hrs.

3.19.10 Diacetyl content in *dahi*

The spectrophotometric method described by Jha *et al*. (2011) was used to detect diacetyl content of *dahi*.

Procedure

1 g *dahi* sample was taken in a stoppered test tube. 0.5 ml of 10% solution of chromotropic acid (in concentrated sulphuric acid) was added to it, 5.0 ml of sulphuric acid (98%) was added drop wise. After vortexing, sample tubes were heated in a water bath at 100ºC for 1 hr and cooled to room temperature. After heat treatment, the contents were transferred to 50 ml volumetric flask and volume was made up to the mark with distilled water. The contents were filtered through Whatman filter paper no. 41. 1 ml was taken from the filtrate, transferred to 25 ml volumetric flask and volume was made up to the mark with the help of distilled water. For preparation of standard curve, standard solutions of diacetyl were prepared in distilled water (0.05, 0.1, 0.15, 0.2 and 0.25 mg/g) and were given the same treatment as in case of *dahi* sample, however, the dilution step was omitted. Optical density of solutions was measured at 570 nm in a spectrophotometer and a standard curve was drawn. The concentration of the diacetyl in *dahi* samples were then interpolated from the diacetyl standard curve (figure 3.8) and corrected for the sample dilution factor.

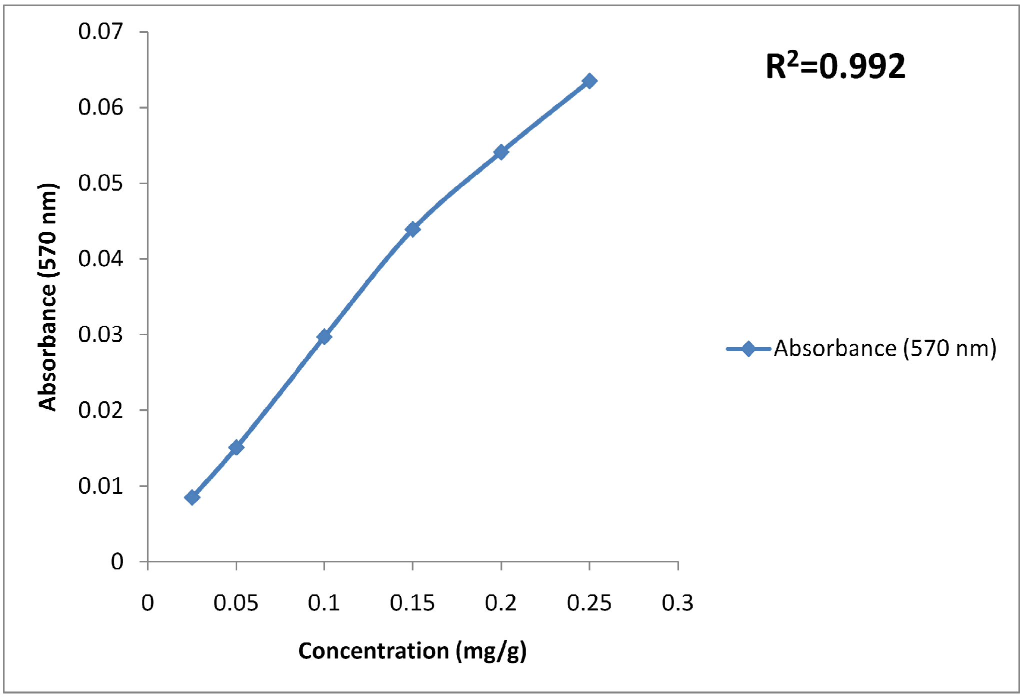

Figure 3.8: Standard curve for estimation of diacetyl content

3.20 Effect of fortification on sensory, physical and microbiological properties of yoghurt

3.20.1 Preparation of yoghurt from fortified milk

Yoghurt was prepared from control and fortified milk samples by the method of Rasic and Kurman (1978). The key steps were standardisation of mix, heat treatment, cooling to incubation temperature, inoculation with yogurt cultures, incubation, cooling and packaging (figure 3.9). Fresh cow and buffalo whole milks were mixed in 1:1 ratio and fat was separated to prepare toned milk. Skim milk powder was added @1% by weight of milk to adjust the solid contents. Iron salts (@15, 20 and 25 ppm iron) and vitamin A acetate (@2500 IU/L) were added to milk and it was heated to 90ºC for 60 minutes. Milk was then cooled and starter culture was added (@1.25% by weight *Streptococcus thermophilus* and 1.25% by weight *Lactobacillus bulgaricus*) and incubated at 42ºC for 5-6 hrs. After the formation of a firm coagulum, yoghurt was cooled to 2-4ºC and stored under refrigeration conditions (4-7ºC). The flow diagram of the steps involved is given below:

Preparation of mix: Separation of fat (toned milk) and standardisation of solids content via addition of non-fat dry milk (@1% by weight)

↓

Pasteurisation: 90ºC for 60 minutes, cool to 45ºC

↓

Inoculation: Add 1.25% by weight of active culture of *Streptococcus thermophilus* and 1.25% of *Lactobacillus delbrueckii* ssp. *bulgaricus*

↓

Incubation at 42ºC for 5-6 hrs

↓

Cool to 4ºC

↓

Packaging and storage

Figure 3.9: Flow chart for manufacture of yoghurt

Sensory analysis, setting time, acidity, water holding capacity, viscosity and texture profile analysis were conducted as described for *dahi.*

3.20.2 Enumeration of *Streptopcoccus thermophilus* and *Lactobacillus bulgaricus* (IDF 1993)

Reagents, diluents and culture media

1. M17 for enumeration of *Streptococcus thermophilus*
2. Acidified MRS for enumeration of *Lactobacillus delbrueckii ssp. bulgaricus.*
3. Acetic acid for addition to MRS agar (to reduce pH to 5.4±1) after sterilisation.
4. 10% lactose solution for addition to molten M17 (add 5 ml of a sterile 10% lactose solution to 95 ml of M17).
5. 0.1% non fat dry milk dilution blanks (add 0.1 g/100 ml water)

Procedure

The external surface was aseptically cleaned with a 70% (v/v) solution of ethanol prior to opening the yoghurt sample. The sample was opened and the contents were mixed carefully with a sterile spatula. 10.0±0.1 g of sample was weighed into appropriate container and serial dilution blanks were prepared containing 0.1% nonfat dry milk (0.1 g/100 ml water) upto sixth dilution. Decimal dilutions were prepared in duplicate (for *S. thermophilus* and *L. bulgaicus* ssp. *delbrueckii*) and 1 ml was transferred using a sterile pipette into appropriately labeled petri dishes. 15 ml of appropriate media (acidified MRS for *L. delbrueckii*, M17 with 10% lactose for *S. thermophilus*) was poured into the appropriate petri dish. The contents were mixed carefully by swirling and allowed to solidify. Acidified MRS was incubated anaerobically in inverted position at 37ºC for 72 hrs. M17 was incubated inverted at 37ºC for 48 hrs. After the appropriate incubation time, colonies on the plates (containing between 30 and 300 colonies) were counted.

3.20.3 Acetaldehyde content in yoghurt

Acetaldehyde is the principal flavour component in yoghurt produced by the microbial action on milk components. Megazyme assay kit was used for estimation of acetaldehyde in yoghurt made from control and fortified milks.

Principle

Acetaldehyde is quantitatively oxidised to acetic acid in the presence of aldehyde dehydrogenase (Al-DH) and nicotinamide-adenine dinucleotide (NAD^+).

$$\text{Acetaldehyde} + NAD^+ + H_2O \xrightarrow{\text{(Al-DH)}} \text{acetic acid} + NADH + H^+$$

The amount of NADH formed in this reaction is stoichiometric with the amount of acetaldehyde. It is the NADH that is measured by the increase in optical density at 340 nm.

Preparation of reagent solutions

Solution 1: Buffer (pH 9.0) plus sodium azide (0.02% w/v) as a preservative.

Solution 2: NAD^+ (freeze dried powder): the contents were dissolved in 11.0 ml of distilled water and stored at -20ºC in polypropylene tubes after use.

Solution 3: Aldehyde dehydrogenase solution

Solution 4: Acetaldehyde control powder (acetaldehyde ammonia trimer powder): 80 mg of this powder (~50 mg of acetaldehyde) was dissolved in 1 L distilled water and stored in Duran bottle. This solution was prepared to assay where there was some doubt about the accuracy of the spectrophotometer being used or where it was suspected that inhibition was being caused by substances in the sample.

Sample preparation

40 g yoghurt was weighed accurately into a beaker and 4 ml solution of citric acid (20%, w/v) was added to it. The contents were stirred slightly and transferred to a 50 ml volumetric flask with water and filled up to the mark with water. The contents were shaken and mixed well and filtered through Whatman filter paper no. 42. The clear solution was used for the assay. The main steps involved in acetaldehyde estimation are listed in table 3.2.

Table 3.2: Procedure for acetaldehyde estimation

<table>
<tr><th>Pipette into curettes</th><th>Blank</th><th>Sample</th></tr>
<tr><td>Distilled water
Sample solution
Buffer 1 solution
Solution 2 (NAD$^+$)</td><td>2.10 ml
-
0.20 ml
0.20 ml</td><td>2.00 ml
0.10 ml
0.20 ml
0.20 ml</td></tr>
<tr><td colspan="3">Contents were mixed by gentle inversion, the optical density of the solutions (A_1) was read after approximately 2 minutes and the reactions were started by addition of:</td></tr>
<tr><td>Solution 3 (Al-DH)</td><td>0.05 ml</td><td>0.05 ml</td></tr>
<tr><td colspan="3">Contents were mixed by gentle inversion, the optical density of the solutions (A_2) were read at the end of the reaction (approx. 3-4 minutes). If the reaction had not stopped after 3-4 minutes, optical densities were continuously read at 2 minutes intervals until the optical density increased constantly over 2 minutes.</td></tr>
</table>

Calculation

The optical density difference (A_2-A_1) was determined for both blank and sample. The optical density difference of the blank was subtracted from the optical density difference of the sample, thereby obtaining $\Delta A_{acetaldehyde}$.

The concentration of acetaldehyde can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{acetaldehyde} \quad (g/L)$$

where, V = final volume (ml)

MW = molecular weight of acetaldehyde (g/mol)

ε = extinction coefficient of NADH at 340 nm= 6300 ($l \times mol^{-1} \times cm^{-1}$)

d = light path (cm)

v = sample volume (ml)

It follows for acetaldehyde:

$$c = \frac{2.55 \times 44.05}{6300 \times 1 \times 0.10} \times \Delta A_{acetaldehyde} \quad (g/L)$$

$$= 0.1783 \times \Delta A_{acetaldehyde} \ (g/L)$$

$$\text{Content of acetaldehyde} = \frac{C_{acetaldehyde}\ (g/L \text{ sample solution})}{Weight_{sample}\ (g/L \text{ sample solution})} \times 100 \quad (g/100g)$$

3.21 Bioavailability of added nutrients

3.21.1 *In vitro* study

In vitro digestibility i.e. bioaccessibility of control and fortified milk was determined by dialysis method (Herrero-Barbudo *et al.* 2009). Control and fortified toned milk were pasteurised (63°C for 30 minutes) and used for determination of vitamin A and iron digestibility.

Dialysis membrane was dipped in deionised water for 30 minutes before use. The composition of organic and inorganic solutions is given in table 3.3. Approximately, 15 ml milk samples (control and fortified) were transferred to a flask and a saliva solution (9 ml, pH 6.5) containing organic and inorganic components and α-amylase (700 mg/L) was added after which the samples were incubated in a shaking water bath at 37°C, 95 rpm for 5 minutes. Gastric juice (13.5 ml) with organic and inorganic solutions, mucin (3 g/L gastric juice), bovine serum albumin (1 g/L), and pepsin (1 g/L) from porcine stomach was added. pH was adjusted to 1.1 with HCl and the solution was incubated for 1 hr. Duodenal juice (25 ml, organic plus inorganic solutions, containing 3 g/L porcine pancreatin) and bile solutions (9 ml, containing 6 g/L bovine bile), prepared on the day of assay, were introduced after neutralisation of the pH (7.8) and the human pancreatic lipase (5 units), colipase (12.5 mg), cholesterol esterase (5 units), phospolipase A_2 (50 μl) and taurocholate salts (19.9 mg) were added. Final volume was approximately 70 ml, the mixture was then incubated up to 3 hrs at 37°C. Transfer from the duodenal digesta to the aqueous-micellar phase was estimated by calculating vitamin A and iron in the supernatants after decantation for 16 hrs at room temperature. Dialysis sac was formed by tying the membrane with thread on one side, the digested contents thus obtained were poured in this dialysis sac and sac was tied from opposite side also. The contents alongwith this dialysis sac were dipped in distilled water kept in a 1000 ml beaker overnight. Vitamin A and iron content of retentate and permeate were determined by HPLC and AAS to estimate the digestibility of the added nutrients under simulated gastro-intestinal conditions.

Calculation

The bioavailability of the nutrient i.e. bioaccessibility was calculated from the amount of the nutrient (vitamin A or iron) that had passed the dialysis membrane proportional to the total nutrient (vitamin A or iron) content of the sample. The following equation was used:

Bioaccessibility (%) = D/C x 100

where, D = vitamin A or iron content in the dialysate and

C = vitamin A or iron content of sample

Table 3.3: Composition and concentration of the various synthetic juices of the *in vitro* digestion

Solution↓	**Saliva**	**Gastric Juice**	**Duodenal juice**	**Bile**
Inorganic solution	10 ml KCl (89.6 g/L) 10 ml KSCN (20 g/L) 10 ml NaH_2PO_4 (88.8 g/L) 10 ml Na_2HPO_4 (57 g/L) 1.7 ml NaCl (175.3 g/L) 1.8 ml NaOH (40 g/L)	15.7 ml NaCl (175.3 mg/L) 3.0 ml NaH_2PO_4 (88.8 mg/L) 9.2 ml KCl (89.6 g/L) 18 ml $CaCl_2$. 2 H_2O (22.2 mg/L) 10 ml NH_4Cl (30.6 g/L) 8.3 ml HCl (37%)	40 ml NaCl (175.3 mg/L) 10 ml $NaHCO_3$ (84.7 mg/L) 10 ml KH_2PO_4 (8 g/L) 6.3 ml KCl (89.6 g/L) 10 ml $MgCl_2$ (5 mg/L) 180 µl HCl (37%)	30 ml NaCl (175.3 g/L) 68.3 ml $NaHCO_3$ (84.7 g/L) 4.2 ml KCl (89.6 g/L) 200 µl HCl (37%)
Organic solution	8 ml urea (25 g/L)	10 ml glucose (65 g/L) 10 ml glucuronic acid (2 g/L) 3.4 ml urea (25 g/L) 10 ml glucosamine hydrochloride (33 g/L)	4 ml urea (25 g/L)	10 ml urea (25 g/L)
Add to mixture organic+ inorganic solution	145 mg α-amylase 15 mg uric acid 50 mg mucin	1 g BSA 1 g pepsin 3 g mucin	9 ml $CaCl_2$. 2 H_2O (22.2 g/L) 1 g BSA 3 g pancreatin Pancreatic lipase (5 units) 12.5 µg colipase Cholesterol esterase (5 units) 50 µl phospholipase A_2 19.9 mg sodium taurocholate	10 ml $CaCl_2$. 2 H_2O (22.2 g/L) 1.8 g BSA 6 g bile
pH	6.5±0.2	1.07±0.07	7.8±0.2	8.0±0.2
The inorganic and organic solutions were augmented to 500 ml with distilled water.				

3.21.2 *In vivo* study

3.21.2.1 Animals

Male albino rats of Wistar strain used in the study were procured from Central Animal Facility, National Institute of Pharmaceutical Education and Research (NIPER), Mohali, Punjab, India. The present study was approved by the Institutional Animal Ethics Committee (IAEC), NDRI, Karnal, India. Specifications are as follows:

Species/Common name	:	Rats, Wistar albino
Weight/Age	:	55-80 g, weaning
Number of animals	:	48+24

From the start of the study, the animals were placed in individual metabolic cages in an environmentally controlled room with a constant temperature of 20–22ºC, a 12 hrs light-dark cycle and 55-60% humidity in a well ventilated room in Small Animal House, NDRI, Karnal, India.

3.21.2.2 Diet composition

During the initial seven days, rats were fed with basal diet (table 3.4) obtained from Small Animal House, NDRI, Karnal in order to provide an acclimatisation period. Thereafter, during the experimental period, they were provided with de-ionised water and synthetic diet as the composition (table 3.5) in which almost 33% replacement was done with control and fortified milk lyophilates. Composition of mineral and vitamin mixture used in. normal basal diets is given in table 3.6 and 3.7, respectively. One group (anaemic) was given a pre-experimental period of 21 days and was fed with anaemic i.e. low iron diet (table 3.5) in order to induce dietary iron deficiency, thereafter, it was fed with normal diet and lyophilates of control and fortified milks.

Table 3.4: Composition of basal diet per 100.4 kg given during acclimatisation period

Composition	Quantity (kg)
Wheat crushed	15.0
Bengal gram crushed	58.0
Ground nut cake crushed	10.0
Refined oil	4.0
SMP	5.0
Casein	4.0
Salt mixture	4.0
Vitamin mixture	0.2
Choline chloride mixture	0.2

Table 3.5: Composition of normal diet (for control and anaemic group) and protein free diet

Ingredient	%
Starch	59.00% for control group 62.00% for anaemic group
Fat	10
Protein	16.00% for control and anaemic group 0.00% protein for protein free diet
Cellulose	2.50
Sucrose	7.50
*Mineral mixture	3.50
*Vitamin mixture	1.00
Choline chloride	0.25
DL methionine	0.30
Final weight was made up to 1 kg with starch	

*Mineral and vitamin mixture were prepared and mixed according to AOAC (1995)

Table 3.6: Composition of mineral mixture (g/kg)

Mineral salt	**Quantity (g/kg)**
KH_2PO_4	389.00
$MgSO_4$	57.30
$CaCO_3$	381.40
$MnSO_4.7H_2O$	4.10
$CuSO_4.5H_2O$	0.477
$CoCl_4.6H_2O$	0.023
NaCl	139.3
KI	0.79
$ZnSO_4.7H_2O$	0.55
$FeSO_4.7H_2O$	0.224 g for control and protein free diet 0.025 g for anaemic diet
Final weight was made up to 1 kg with starch	

Table 3.7: Composition of vitamin mixture (mg/100 g)

Vitamin	**Quantity (per 100g)**
Vitamin A	600.00 µg
Vitamin D	5.00 µg
Vitamin E	6.71 mg
Choline chloride	200.00 mg
Inositol	10.00 mg
PABA	10.00 mg
Niacin	4.00 mg
Ca-D Pentothenate	4.00 mg
Vitamin K	0.50 mg
Riboflavin	0.80 mg
Thiamine hydrochloride	0.50 mg
Biotin	0.04 mg
Folic acid	0.20 mg
Pyridoxine HCl	0.50 mg
Vitamin B_{12}	0.003 mg
Final weight was made up to 100 g with starch	

3.21.2.3 Experimental design

Rats were randomly divided into two groups, each group containing 24 rats. One group was maintained as the normal group which was again subdivided in 3 groups: 1) control, 2) iron fortified and 3) iron+vitamin A fortified. Control group was subjected to an experimental period of 21 days. They were fed with synthetic normal diet+control milk lyophilates (unfortified) and those in iron fortified group were provided with normal diet+iron fortified milk lyophilates. Iron+vitamin A fortified group was fed with normal diet+lyophilates of milk fortified with vitamin A and iron. Blood was collected from this group at zero day as well on the 21st day and analysed for haemoglobin content, plasma ferritin content and plasma transferrin content. All the animals were sacrificed at the end of 21 days experimental period and their liver was collected.

The other group i.e. anaemic group was subjected to a pre-experimental period of 21 days in which they were provided with synthetic anaemic diet. After ensuring the occurrence of anaemia, the test group was subjected to the same grouping and feeding pattern as mentioned for normal group. Blood was collected at 0 day, 21st day and 42nd day and the samples were subjected to analysis. Diet and de-ionised water were available *ad libitum* to all rats. For determination of digestive and metabolic utilisation of iron and vitamin A, balance (absorption-retention) studies were conducted for three days during the experimental period for which food intake was measured and urine and faeces collected daily. Figure 3.10 represents the experimental design of *in vivo* study.

To study the effect of added micronutrients (iron and vitamin A) on the digestibility of milk proteins, 24 male albino wistar rats were selected having same age and body weight and were divided into three groups:

1. Non-protein group: fed with protein free diet
2. Control group: fed with normal diet+unfortified milk lyophilate
3. Iron+vitamin A fortified group: fed with normal diet+vitamin A and iron fortified milk lyophilates

The animals were fed with the respective diets for 10 days. Urine and faeces of the rats were collected daily by keeping the rats in metabolic cages and their nitrogen content was estimated by Kjeldahl method (AOAC 1984) and the data was used to calculate the protein quality parameters (digestibility coefficient, biological value and net protein utlisation). The experimental design of study is shown in figure 3.11.

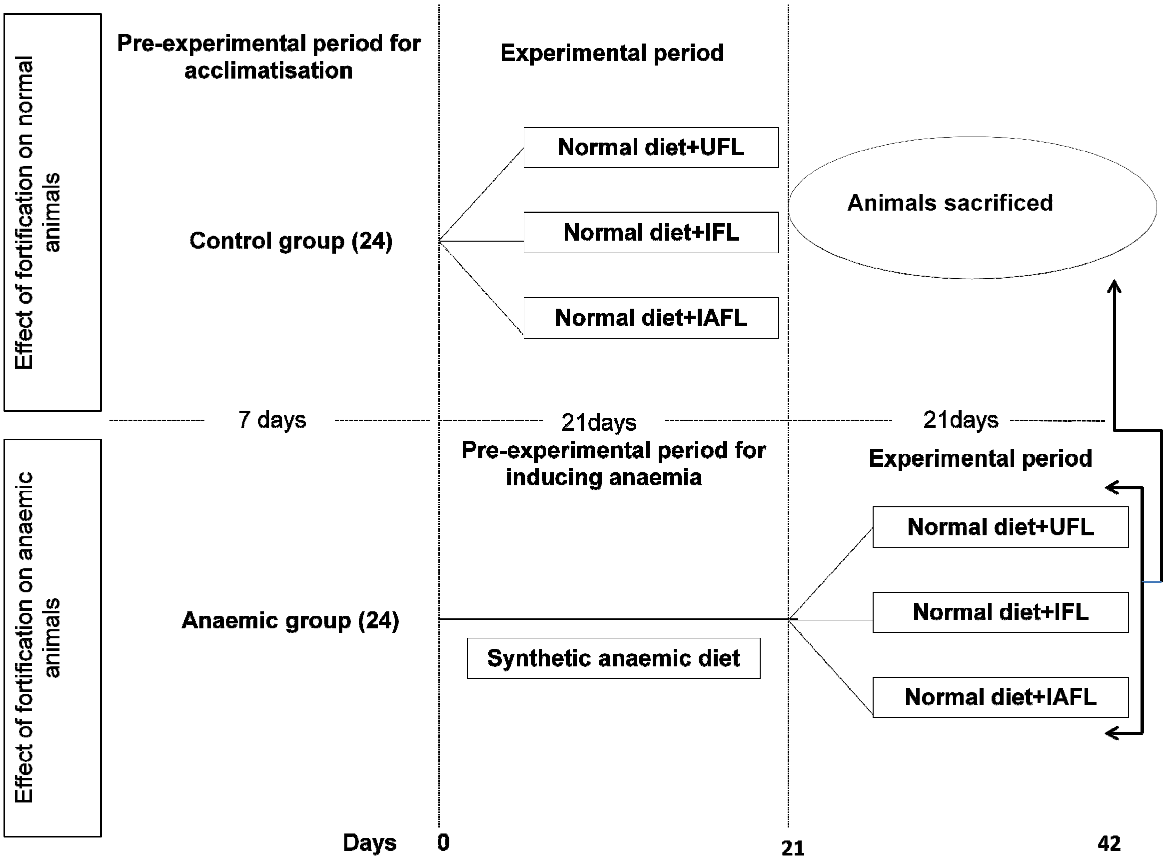

Figure 3.10: Experimental design of *in vivo* study

where, UFL = unfortified milk lyophilates

IFL = iron fortified milk lyophilates

IAFL = iron+vitamin A fortified milk lyophilates

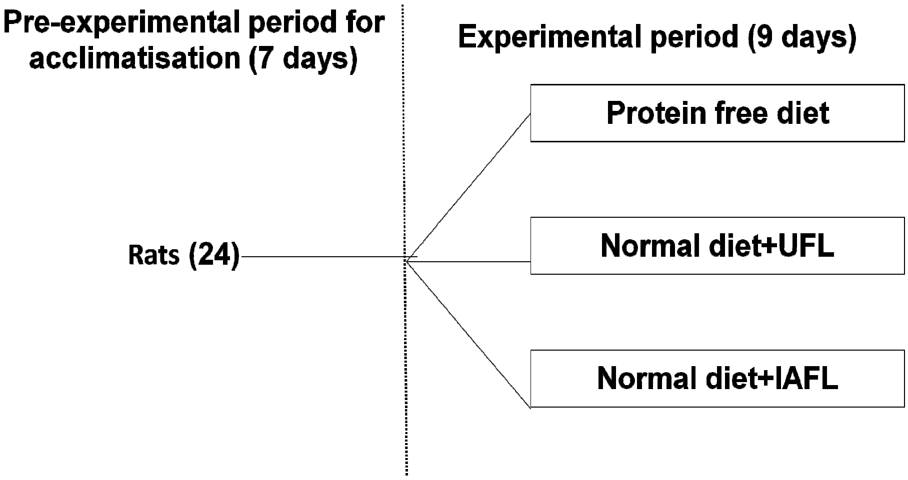

Figure 3.11: Experimental design for estimation of protein quality parameters

3.21.2.4 Iron and vitamin A content of diet given to rats (normal diet+lyophilates)

Table 3.8 shows iron and vitamin A content per 15 g diet (normal diet+lyophilates).

10 g normal diet contains = 0.45 mg iron

5 g unfortified milk lyophilates contain = 0.03 mg iron

5 g iron and iron+vitamin A fortified milk lyophilates contain = 6.25 mg iron

10 g normal diet contains = 0.6 μg vitamin A

5 g unfortified and iron fortified milk lyophilates contain = 0.601 μg vitamin A

5 g iron+vitamin A fortified milk lyophilates contain = 206.25 μg vitamin A

Diet given to control group rats per day (15 g): Normal diet (10 g)+5 g milk lyophilates

Group A: 10 g normal diet + 5 g unfortified milk lyophilates

Group B: 10 g normal diet + 5 g iron fortified milk lyophilates

Group C: 10 g normal diet + 5 g iron+vitamin A fortified milk lyophilates

Table 3.8: Vitamin A and iron intake of rats per 15 g diet daily

Group	Vitamin A intake per 15 g diet (μg)	Iron intake per 15 g diet (mg)
Unfortified	1.201	0.48
Iron fortified	1.201	6.73
Iron+vitamin A fortified	207.25	6.73

3.21.2.5 Biological indices

Biological indices namely apparent digestibility coefficient and % retention/intake of iron and vitamin A were estimated to check their digestive and metabolic utilisation. During the experimental period of three days, food intake was measured and urine and faeces were collected daily. Faeces samples were taken in a crucible and ashing was done at 500ºC for 4 hrs in a muffle furnace. After cooling, the ash was dissolved in 5 ml of 6 M HCl and volume was made up to 50 ml using double distilled water for iron analysis using AAS. Urine was collected in a 0.5% v/v HCl solution and later filtered and analysed for iron content. Similarly, faeces and urine samples were saponified at 70ºC for 30 minutes and same extraction and HPLC analysis procedure was followed as described in section 3.10.2 and 3.10.3 for milk. The parameters were calculated as follows:

$$\text{Apparent digestibility coefficient} = \frac{(I-F) \times 100}{I}$$

$$\text{Balance retention} = I-(F-U)$$

$$\text{\%Retention/Intake} = \frac{[I-(F-U)] \times 100}{I}$$

where, I = intake of iron

F = faecal excretion of iron

U = urinary excretion of iron

3.21.2.6 Haemoglobin repletion assay

Haemoglobin, the main component of the red blood cell, functions in the transportation of oxygen and CO_2. Haemoglobin consists of one molecule of globin and 4 molecules of haeme (each containing one molecule of iron in the ferrous state). Haemoglobin estimation was done according to cyanomethaemoglobin method outlined by Drabkin and Austin (1932).

Principle

The principle of this method is that when blood is mixed with Drabkin's reagent (a solution containing potassium ferricyanide, potassium cyanide and sodium bicarbonate), the potassium ferricyanide then combines with methaemoglobin to from cyanomethaemoglobin, which is a stable colour pigment read photometrically at a wave length of 540 nm.

Procedure

Blood samples were collected in vaccutainers internally coated with heparin. Reagents were added as explained in table 3.9:

Table 3.9: Procedure for the estimation of haemoglobin concentration

Sample	Drabkin's reagent	Cyanomethaemoglobin standard	Distilled water	Blood
Blank	5 ml	-	20 µl	-
Standard	-	5 ml	-	-
Blood sample	5 ml	-	-	20 µl

The samples were kept for 10 minutes incubation at room temperature. The optical density was then read at 540 nm. The haemoglobin content was calculated as:

$$\text{Haemoglobin (g/dl)} = \frac{\text{OD test}}{\text{OD standard}} \times 15$$

3.21.2.7 Plasma ferritin

Ferritin is a water soluble, iron storage protein. Ferritin levels are an indicator of the amount of iron absorbed in the body. The ferritin level in the rat plasma samples was measured using highly sensitive two site enzyme linked immunoassay (ELISA) kit.

Principle

The principle of the double antibody sandwich ELISA is represented in figure 3.12. In this assay, the ferritin present in sample reacts with the anti-ferritin antibodies which have been adsorbed to the surface of polystyrene microtitre wells. After the removal of unbound proteins by washing, anti-ferritin antibodies conjugated with horseradish peroxidise (HRP), are added. These enzyme-labelled antibodies form complexes with the previously bound ferritin. Following another washing step, the enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate, 3,3',5,5'- tetramethylbenzidine (TMB). The quantity of bound enzyme varies directly with the concentration of ferritin in the sample tested; thus, the optical density, at 450 nm, is a measure of the concentration of ferritin in the test sample.

Anti-ferritin antibodies bound to solid phase
↓
Standards and samples added
↓
Ferritin*anti-ferritin complexes formed
↓
Unbound sample proteins removed
↓
Anti-ferritin-hrp conjugate added
↓
Anti-ferritin-HRP* ferritin * anti-ferritin complexes formed
↓
Unbound anti-ferritin-HRP removed
↓
Chromogenic substrate added
↓
Bound enzyme activity determined

Figure 3.12: Principle of double body sandwich ELISA for ferritin

Reagents

1. Diluent concentrate

The diluent solution supplied was a 5X concentrate and was diluted 1:5 with distilled water (1 part buffer concentrate, 4 parts distilled water).

2. Wash solution concentrate

The wash solution supplied was a 20X concentrate and was diluted 1:20 with distilled water (1 part buffer concentrate, 19 parts distilled water).

3. Enzyme-antibody conjugate

The required amount of working conjugate solution was prepared for each microtitre plate test strip by adding 10 μl enzyme-antibody conjugate 100X (affinity purified anti-ferritin antibody conjugated with horseradish peroxidase in a stabilising buffer) to 990 μl of 1X diluent for each test strip to be used for testing.

4. Chromogen-substrate solution

The reagent supplied was 3,3’,5,5’-tetramethybenzidine (TMB) and hydrogen peroxide in citric acid buffer at pH 3.3.

5. Rat ferritin calibrator

The rat ferritin calibrator was having a concentration of 2.7 µg/ml. Rat ferritin standards were prepared immediately prior to use as per table 3.10:

Table 3.10: Preparation of ferritin standard solutions of different concentrations

Standard	ng/ml	Volume added to 1X diluent	Volume of 1X diluent
6	400	100 µl of rat ferritin calibrator	575 µl
5	200	300 µl of standard 6	300 µl
4	100	300 µl of standard 5	300 µl
3	50	300 µl of standard 4	300 µl
2	25	300 µl of standard 3	300 µl
1	12.5	300 µl of standard 2	300 µl
0 (Blank)	0	-	500 µl

6. Anti-rat ferritin ELISA micro plate

7. Stop solution

The reagent supplied was 0.3 M sulphuric acid and it was ready to use as supplied in the kit.

Sample collection and handling

Blood was collected by venipuncture in a vaccutainer internally coated with heparin and then centrifuged at 1500 rcf for 15 minutes at 4ºC. The supernatant plasma layer was transferred to another vial and stored at -20ºC.

Sample dilution

A 1:100 dilution of sample was prepared. 5 µl sample was taken and made upto 500 µl using diluent buffer and mixed thoroughly.

Assay procedure

All reagents were brought to room temperature before the assay. 100 µl of all the standards were pipetted in duplicate in the designated wells in the provided microplate. 100 µl of pre-diluted sample were pipetted into wells. The microplate was incubated at room temperature for sixty (60±2) minutes. The plate was covered and kept in level during incubation. Following incubation, the contents of the wells were aspirated. Each well was filled with appropriately diluted wash solution and aspirated. This was repeated three times, a total of four washes and the plates were struck on the absorbent paper to remove residual buffer. 100 µl of appropriately diluted enzyme-antibody conjugate was added to each well. Incubation was done at room temperature for ten (10±2) minutes. The plates were covered and kept in level and dark during incubation. Washing and blotting steps were repeated as before. 100 µl of TMB substrate solution was pipetted into each well. The plates were incubated again in the dark at room temperature for precisely ten (10) minutes, after which 100 µl of stop solution was added to each well. The optical density (450 nm) of the contents of each well was measured using an automated ELISA plate reader. The plate reader was calibrated to air. A standard curve (polynomial) was constructed (figure 3.13) and the test values were interpolated from the standard curve. The values were corrected for dilution.

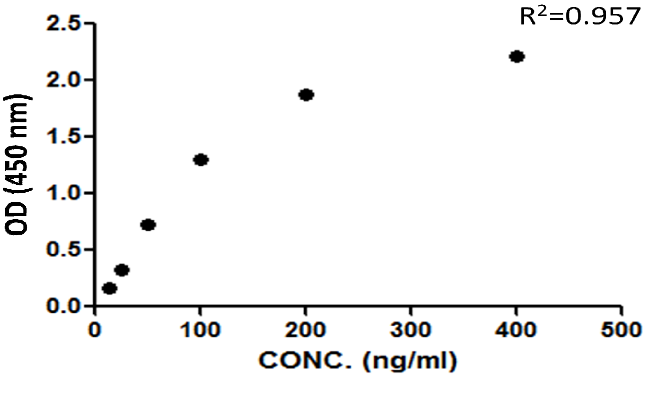

Figure 3.13: Standard curve for estimation of ferritin content

3.21.2.8 Plasma transferrin

Transferrin (TX) is a metal-combining protein that reversibly binds to acid soluble iron in plasma. It functions to transport iron to the bone marrow and to tissue storage organs such as the liver. Transferrin also participates in the regulation and control of iron absorption and protects against iron intoxication.

Principle

The principle of the double antibody sandwich ELISA is represented in figure 3.14. In this assay, the transferrin (TX) present in sample reacts with the anti-transferrin antibodies which have been adsorbed to the surface of polystyrene microtitre wells. After the removal of unbound proteins by washing, anti-TX antibodies conjugated with horseradish peroxidise (HRP), are added. These enzyme-labelled antibodies form complexes with the previously bound TX. Following another washing step, the enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB). The quantity of bound enzyme varies directly with the concentration of TX in the sample tested; thus, the optical density, at 450 nm, is a measure of the concentration of TX in the test sample.

Anti-TX antibodies bound to solid phase
↓
Standards and samples added
↓
TX*anti-TX complexes formed
↓
Unbound sample proteins removed
↓
Anti-TX-HRP conjugate added
↓
Anti-TX-HRP*TX*anti-TX complexes formed
↓
Unbound anti-TX-HRP removed
↓
Chromogenic substrate added
↓
Bound enzyme activity determined

Figure 3.14: Principle of double body sandwich ELISA for transferrin

Reagents

1. Diluent concentrate

The diluent solution supplied was a 5X concentrate and was diluted 1:5 with distilled water (1 part buffer concentrate, 4 parts distilled water).

2. Wash solution concentrate

The wash solution supplied was a 20X concentrate and was diluted 1:20 with distilled water (1 part buffer concentrate, 19 parts distilled water).

3. Enzyme-antibody conjugate

The required amount of working conjugate solution was prepared for each microtitre plate test strip by adding 10 µl enzyme-antibody conjugate 100X (affinity purified anti-transferrin antibody conjugated with horseradish peroxidase in a stabilising buffer) to 990 µl of 1X diluent for each test strip to be used for testing.

4. Chromogen-substrate solution

The reagent supplied was 3,3',5,5'-tetramethybenzidine (TMB) and hydrogen peroxide in citric acid buffer at pH 3.3.

5. Rat transferrin calibrator

1.0 ml of distilled water was added to the rat transferrin calibrator and mixed gently until dissolved. The calibrator was then at a concentration of 10.56 µg/ml (the reconstituted calibrator was aliquoted and kept in frozen condition for future use). Rat transferrin standards were prepared immediately prior to use as per table 3.11:

Table 3.11: Preparation of transferrin standard solutions of different concentrations

Standard	ng/ml	Volume added to 1X diluent	Volume of 1X diluent
7	400	36 µl of reconstituted rat TX calibrator	914 µl
6	200	300 µl of standard 7	300 µl
5	100	300 µl of standard 6	300 µl
4	50	300 µl of standard 5	300 µl
3	25	300 µl of standard 4	300 µl
2	12.5	300 µl of standard 3	300 µl
1	6.25	300 µl of standard 2	300 µl
0 (Blank)	0	-	500 µl

6. Anti-rat transferrin ELISA micro plate

7. Stop solution

The reagent supplied was 0.3 M sulphuric acid and it was ready to use as supplied in the kit.

Sample collection and handling

Blood was collected by venipuncture in a vaccutainer internally coated with heparin and then centrifuged at 1500 rcf for 15 minutes at 4ºC. The supernatant plasma layer was transferred to another vial and stored at -20ºC.

Sample dilution

A 1:200000 dilution of sample was prepared. 5 µl blood plasma sample was added to 995 µl of 1X diluent, this was 200 times dilutes sample. 4 µl of this diluted sample was taken and added to 4996 µl of 1X diluent. The final sample thus obtained was having a dilution of 200000 times.

Assay procedure

All reagents were brought to room temperature before the assay. 100 µl of all the standards were pipetted in duplicate in the designated wells in the provided microplate. 100 µl of pre-diluted sample were pipetted into wells. The microplate was incubated at room temperature for fifteen (15±2) minutes. The plate was covered and kept in level during incubation. Following incubation, the contents of the wells were aspirated. Each well was filled with appropriately diluted wash solution and aspirated. This was repeated three times, a total of four washes and the plates were struck on the absorbent paper to remove residual buffer. 100 µl of appropriately diluted enzyme-antibody conjugate was added to each well. Incubation was done at room temperature for fifteen (15±2) minutes. The plates were covered and kept in level and dark during incubation. Washing and blotting steps were repeated as before. 100 µl of TMB substrate solution into was pipetted into each well. The plates were again incubated in the dark at room temperature for precisely ten (10) minutes, after which 100 µl of stop solution was added to each well. The optical density (450 nm) of the contents of each well was measured using an automated ELISA plate reader. The plate reader was calibrated to air. A standard curve (polynomial) was constructed (figure 3.15) and the test values were interpolated from the standard curve and values were corrected for dilution.

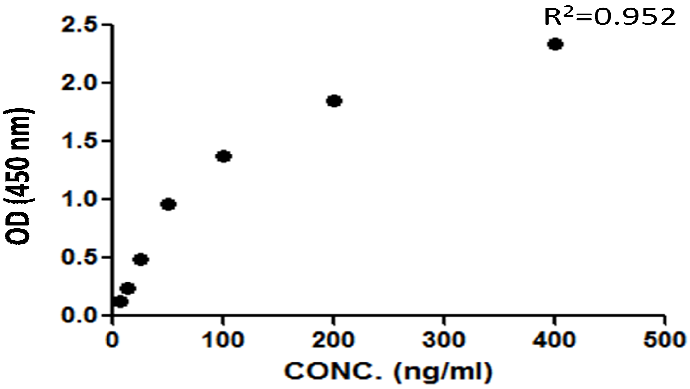

Figure 3.15: Standard curve for estimation of transferrin content

3.21.2.9 Collection of liver

All the animals were sacrificed at the end of 21 days experimental period. They were first anaesthetised using diethyl ether and then killed by cervical dislocation. After this, the rats were dissected and entire liver was excised, washed with isotonic buffer three times and stored at -20^{0}C.

3.21.2.9.1 Estimation of vitamin A and iron in liver

Total vitamin A and iron in liver were determined by AAS and HPLC as described in section 3.10 and 3.11, respectively. In case of vitamin A, ammonium hydroxide and HCl were not added to liver sample since these were incorporated to break the wall material of vitamin A used for milk fortification.

3.21.2.10 Protein quality parameters

From the amino acids absorbed into the blood stream after the process of digestion, the body will utilise a portion of the amino acids required for the repair and growth of various tissues and to meet other physiological requirements. Quantitative data regarding the relative digestibility coefficient and nutritive value of proteins, i.e. suitability to meet the protein requirements of the body can be obtained through experiments on animals and human beings. In this study, rats were selected for estimation of protein quality parameters viz. digestibility coefficient, biological value and net protein utilisation.

3.21.2.10.1 Digestibility coefficient (DC)

Dietary proteins are hydrolysed to amino acids during digestion. The percentage of the ingested protein absorbed into the blood stream after the process of digestion is complete is referred to as digestibility coefficient. When an animal is fed with nitrogen-free diet, certain amount of nitrogen is excreted in the faeces. This is derived mainly from the digestive juices. This is called endogenous faecal nitrogen. When a food is given, the nitrogen found in faeces consists of both of endogenous nitrogen and food nitrogen lost in digestion. To find nitrogen lost in digestion, endogenous faecal nitrogen also needs to be determined.

For the determination of digestibility coefficient, the following data are required:

1) Food nitrogen intake (I_n);
2) Total faecal nitrogen excreted (F_n), and
3) Endogenous faecal nitrogen (F_e)

The digestibility coefficient can be calculated using the following formula:

$$\text{Digestibility coefficient} = 100 \times \frac{\text{N intake-(N in faeces-endogenous faecal N)}}{\text{N intake}}$$

$$= 100 \times \frac{I_n-(F_n-F_e)}{I_n}$$

where, F_n-F_e is the food nitrogen lost in digestion.

3.21.2.10.2 Biological value (BV)

Biological value measures the quantity of dietary proteins utilised by the animal for meeting its protein needs for maintenance and growth.

$$\text{Biological value} = \frac{\text{(N digested-N lost in metabolism)} \times 100}{\text{N digested}}$$

N digested = N intake (I_n) - N in faeces (F_n) on the protein diet - N in faeces (F_e) on protein-free diet

i.e. N digested = $I_n - (F_n - F_e)$

N lost in metabolism = N in urine (U_n) on protein diet – N in urine (U_e) on protein-free diet = $U_n - U_e$

$$\text{Biological value} = \frac{I_n - (F_n - F_e) - (U_n - U_e) \times 100}{I_n - (F_n - F_e)}$$

3.21.2.10.3 Net protein utilisation (NPU)

Net protein utilisation of dietary protein is the product of digestibility coefficient and biological value divided by 100.

$$\text{Net protein utilisation} = \frac{\text{Digestibility coefficient x Biological value}}{100}$$

$$\text{NPU} = \frac{\text{Body N of the test group} - \text{Body N of the non-protein group} + \text{N consumed by non-protein group}}{\text{N consumed by the test group}} \times 100$$

NPU of a food or diet helps in the calculation of the net available protein from a diet.

3.21.2.10.4 Nitrogen estimation

Nitrogen content of the faeces and urine samples was determined by a method described in AOAC (1984). About 200 mg of the faeces was accurately weighed into 100 ml Kjeldahl flask and 20 ml concentrated sulphuric acid were then poured down the neck in such a way as to wash any sample particles on the body of the flask. About 0.2 g of copper sulphate and 10 g of potassium sulphate were then added. The contents of the flask were digested until clear remnant was obtained. It was then cooled and contents of the flask were transferred to a 100 ml volumetric flask and the volume was made up to the mark.10ml of the digest was pipette into the Kjeldahl flask. The tip of the condenser was dipped in a flask containing 10 ml saturated boric acid solution in which mixed indicator (prepared by mixing 1 part of saturated alcoholic methyl red solution and one part of 0.2% alcoholic methylene blue solution) had already been added. About 20 ml 50% sodium hydroxide solution was then added, followed by little amount of distilled water. The distillation was continued till about 50 ml of the distillate was collected in the conical flask. The content of the conical flask was then titrated against 0.02 N hydrochloric acid. A blank determination was also carried out using sucrose instead of sample.

From these values the percentage of total nitrogen in the sample was calculated as follows:

$$\% N = \frac{(T-B) \times N \times 1.401}{\text{Sample weight (g)}}$$

where, T= ml acid required for sample titration

B= ml acid required for blank titration

N= normality of acid used for titration

3.22 Statistical analysis

The results were expressed as means±standard error of mean. Significance was tested by employing single way analysis of variance (ANOVA) and two way analysis of variance (ANOVA) with replication and comparison between means was made by critical difference (CD) value as described by Snedecor and Cochran (1994). For computation of data, software application programme Microsoft Office Excel 2007 was used.

CHAPTER –4

Results and Discussion

4. Results and Discussion

This chapter deals with the various results obtained during the present study. The first phase of this study dealt with the selection of the suitable iron salt and vitamin A ester for fortification. In the second phase, studies were conducted regarding heat stability of fortified milk, stability of added nutrients under different processing conditions and physico-chemical properties. In the third phase, fortified milk was converted into *dahi* and yoghurt and effect of fortification on sensory, physical and microbiological properties of these fermented products was studied. Finally, *in vitro* study under simulated gastro-intestinal conditions; and *in vivo* studies using albino wistar rats were conducted to study the bioavailability of added micronutrients. From the present investigation, results of different experiments are listed and discussed under the following heads:

4.1 Identification of iron salt and vitamin A esters for fortification

The first activity of the study was to select suitable iron salts and vitamin A esters for fortification of milk. Depending on the available information from literature on the characteristics of various iron salts and vitamin A esters, and their availability; nine iron salts (ferrous sulphate heptahydrate, BioFer (microencapsulated ferrous sulphate heptahydrate), ferrous lactate hydrate, ferrous gluconate hydrate, ferric ammonium citrate brown and green, ferric pyrophosphate soluble, ferric chloride hexahydrate and monosodium ferric EDTA) and two vitamin A esters (vitamin A palmitate and vitamin A acetate) were selected for fortification of toned milk. Out of these different iron salts and vitamin A esters, further selection was done based on sensory evaluation, pH, acidity and TBA value. Above 30 ppm level of iron addition, there were noticeable changes in flavour and odour of fortified milk samples; therefore, the fortification levels were restricted to 25 ppm iron. All the iron salts were evaluated at three levels viz. 15, 20 and 25 ppm iron. Vitamin A esters were also evaluated at three levels i.e. 2000, 2500 and 3000 IU/L. Milk was skimmed and fat content was standardised to 3% and solid-not-fat (SNF) to 8.5% to prepare toned milk, which was then fortified with iron salts and vitamin A esters individually and in combination. The fortified milk was then pasteurised at 63ºC/30 minutes and kept under refrigerated conditions (4-7ºC) for 2 hrs. The control and fortified milk samples were subjected to sensory analysis and physico-chemical evaluation viz. pH, acidity and TBA value.

Sensory analysis of control and fortified milk samples was carried out by a team of selected panelists from the institute, on the basis of BIS composite sensory card for milk (Appendix 1). Sensory evaluation was based on four important characteristics i.e. colour and appearance, odour, taste and mouthfeel.

4.1.1 Sensory analysis scores, pH, acidity and TBA value of milk fortified with iron salts

The overall sensory scores obtained for ferric pyrophosphate (FPP) soluble fortified milk samples were 96.29, 96.22 and 96.22 for the added iron concentrations 15, 20 and 25 ppm, respectively as compared to 96.36 obtained for control milk (table 4.1). Statistically, the control and fortified milk samples were non significantly different (P<0.05) in all sensory aspects i.e. colour and appearance, odour, taste, mouthfeel and total scores. Our results were in corroboration with Demott (1971) who also reported that ferric pyrophosphate (added @5.2 and 10.4 ppm iron) caused only minor off-flavours in whole and skim milk.

Table 4.1: Effect of added ferric pyrophosphate soluble at different concentrations on sensory scores

Characteristics	**Maximum score**	**Ferric pyrophosphate soluble**			
		Iron concentration (ppm)			
		Control	**15**	**20**	**25**
Colour and appearance	10	9.57±0.01^{a}	9.57±0.01^{a}	9.57±0.01^{a}	9.57±0.01^{a}
Odour	20	19.19±0.04^{a}	19.16±0.04^{a}	19.11±0.04^{a}	19.11±0.04^{a}
Taste	40	38.60±0.11^{a}	38.57±0.11^{a}	38.53±0.1^{a}	38.51±0.11^{a}
Mouthfeel	30	29.00±0.03^{a}	29.00±0.03^{a}	29.00±0.03^{a}	29.00±0.03^{a}
Total score	100	96.36±0.16^{a}	96.29±0.15^{a}	96.22±0.16^{a}	96.22±0.16^{a}

Data are presented as means±SEM (n=30).
$^{a-b}$Means with the same superscript in a row are not significantly different (P<0.05) from each other.

No change was observed by the sensory panel in natural flavour and odour of milk upon fortification. pH, acidity and TBA values were not adversely affected, however, slight but statistically significant difference in these parameters was observed beyond 20 ppm level of iron addition ($P<0.05$) (table 4.2). However, pH, acidity and TBA values of fortified milk even upto 25 ppm level of iron addition did not show appreciable change compared to control milk.

Table 4.2: Effect of added ferric pyrophosphate soluble at different concentrations on pH, acidity and TBA value of milk

Iron conc. used (ppm)	pH	Acidity (%)	TBA (at 532 nm)
Control	6.88±0.010^{a}	0.139±0.0010^{a}	0.019±0.001^{a}
15	6.88±0.010^{a}	0.139±0.0003^{a}	0.019±0.001^{a}
20	6.88±0.003^{a}	0.139±0.0003^{a}	0.020±0.001^{ab}
25	6.86±0.003^{b}	0.140±0.0003^{b}	0.021±0.001^{b}

Data are presented as means±SEM (n=3).
$^{a\text{-}b}$Means with the same superscript in a column are not significantly different ($P<0.05$) from each other.

The overall sensory scores obtained for ferrous gluconate (FG) hydrate fortified milk samples were 96.67, 96.61 and 96.51 for the added iron concentrations 15, 20 and 25 ppm, respectively as compared to 96.73 obtained for control milk (table 4.3). Statistically, the control and fortified milk samples were non significantly different ($P<0.05$) in terms of colour and appearance, odour, taste, mouthfeel and total scores at all levels of addition. No change was observed by the sensory panel in natural flavour and odour of milk upon fortification.

Table 4.3: Effect of added ferrous gluconate hydrate at different concentrations on sensory scores of milk

Characteristics	Maximum score	Ferrous gluconate hydrate			
		Iron concentration (ppm)			
		Control	**15**	**20**	**25**
Colour and appearance	10	9.73±0.02^{a}	9.73±0.02^{a}	9.73±0.02^{a}	9.71±0.02^{a}
Odour	20	19.57±0.04^{a}	19.55±0.03^{a}	19.54±0.04^{a}	19.51±0.03^{a}
Taste	40	38.53±0.11^{a}	38.53±0.11^{a}	38.48±0.12^{a}	38.45±0.11^{a}
Mouthfeel	30	28.90±0.11^{a}	28.87±0.10^{a}	28.87±0.10^{a}	28.83±0.11^{a}
Total score	100	96.73±0.16^{a}	96.67±0.16^{a}	96.61±0.16^{a}	96.51±0.16^{a}

Data are presented as means±SEM (n=30).
$^{a\text{-}b}$Means with the same superscript in a row are not significantly different ($P<0.05$) from each other.

Slight, however, statistically significant difference was observed in acidity beyond 20 ppm level of addition. However, pH and TBA value differed significantly from control milk even beyond 15 ppm level of addition ($P<0.05$) (table 4.4). However, pH, acidity and TBA values of fortified milk even upto 25 ppm level of addition did not show appreciable change compared to control milk.

Table 4.4: Effect of added ferrous gluconate hydrate at different concentrations on pH, acidity and TBA value of milk

Iron conc. used (ppm)	pH	Acidity (%)	TBA (at 532 nm)
Control	6.73±0.003^{a}	0.134±0.0003^{a}	0.021±0.001^{a}
15	6.73±0.010^{a}	0.134±0.0010^{a}	0.021±0.001^{a}
20	6.72±0.010^{b}	0.135±0.0010^{a}	0.023±0.001^{b}
25	6.70±0.003^{c}	0.137±0.0010^{b}	0.025±0.001^{c}

Data are presented as means±SEM (n=3).
$^{a-b}$Means with the same superscript in a column are not significantly different ($P<0.05$) from each other.

The overall sensory scores obtained for ferrous sulphate heptahydrate fortified milk samples were 94.73, 91.60 and 88.17 for the added iron concentrations 15, 20 and 25 ppm, respectively as compared to 97.80 obtained for control milk (table 4.5). Statistical analysis revealed that the fortified milk samples were significantly different ($P<0.05$) in all attributes (colour and appearance, odour, taste and mouthfeel) from control milk. Metallic taste of iron could be easily perceived by the sensory analysis panel. Edmonson *et al.* (1971) reported that ferrous sulphate addition at 10 ppm resulted in oxidised flavour.

Table 4.5: Effect of added ferrous sulphate heptahydrate at different concentrations on sensory scores of milk

Characteristics	Maximum score	Ferrous sulphate hydrate			
		Iron concentration (ppm)			
		Control	15	20	25
Colour and appearance	10	9.8±0.02^{a}	9.53±0.02^{b}	9.23±0.02^{c}	8.83±0.03^{d}
Odour	20	19.33±0.04^{a}	18.33±0.05^{b}	17.30±0.08^{c}	16.80±0.12^{d}
Taste	40	39.40±0.03^{a}	38.10±0.10^{b}	36.67±0.08^{c}	35.53±0.11^{d}
Mouthfeel	30	29.27±0.01^{a}	28.77±0.03^{b}	28.40±0.05^{c}	27.00±0.13^{d}
Total score	100	97.80±0.07^{a}	94.73±0.14^{b}	91.60±0.18^{c}	88.17±0.37^{d}

Data are presented as means±SEM (n=30).
$^{a-b}$Means with the same superscript in a row are not significantly different ($P<0.05$) from each other.

Slight, however, statistically significant difference was observed in pH and acidity beyond 20 ppm level of addition. However, TBA value differed significantly from control milk even after 15 ppm level of addition ($P<0.05$) (table 4.6), indicating that addition of this iron salt led to enhanced oxidation of milk fat.

Table 4.6: Effect of added ferrous sulphate heptahydrate at different concentrations on pH, acidity and TBA value of milk

Iron conc. used (ppm)	pH	Acidity (%)	TBA (at 532 nm)
Control	6.82 ± 0.02^{a}	0.133 ± 0.001^{a}	0.022 ± 0.002^{a}
15	6.80 ± 0.01^{ab}	0.135 ± 0.002^{a}	0.027 ± 0.001^{a}
20	6.78 ± 0.02^{ab}	0.138 ± 0.001^{ab}	0.045 ± 0.003^{b}
25	6.76 ± 0.02^{b}	0.143 ± 0.003^{b}	0.052 ± 0.004^{c}

Data are presented as means±SEM (n=3).
$^{a\text{-}b}$Means with the same superscript in a column are not significantly different ($P<0.05$) from each other.

The overall sensory scores obtained for ferrous lactate hydrate fortified milk samples were 91.87, 86.53 and 82.67 for the added iron concentrations 15, 20 and 25 ppm, respectively as compared to 95.87 obtained for control milk (table 4.7). Statistically, there was significant difference ($P<0.05$) in all sensory attributes (colour and appearance, odour, taste and mouthfeel) of control and fortified milk samples. The total sensory scores of fortified milk at all levels of salt addition were also significantly different ($P<0.05$) from that of control milk. The colour of the fortified milk was slightly greenish at 25 ppm level of addition. Moreover, sour taste was pronounced in all the fortified samples which was absent in case of control milk.

Table 4.7: Effect of added ferrous lactate hydrate at different concentrations on sensory scores of milk

Characteristics	**Maximum score**	**Ferrous lactate hydrate**			
		Iron concentration (ppm)			
		Control	**15**	**20**	**25**
Colour and appearance	10	9.70 ± 0.02^{a}	9.27 ± 0.06^{b}	8.83 ± 0.07^{c}	8.53 ± 0.07^{d}
Odour	20	19.10 ± 0.04^{a}	18.57 ± 0.09^{b}	16.47 ± 0.14^{c}	16.30 ± 0.17^{c}
Taste	40	38.47 ± 0.10^{a}	36.53 ± 0.18^{b}	34.43 ± 0.26^{c}	32.03 ± 0.2^{d}
Mouthfeel	30	28.60 ± 0.11^{a}	27.50 ± 0.13^{b}	26.80 ± 0.28^{c}	25.80 ± 0.24^{d}
Total score	100	95.87 ± 0.23^{a}	91.87 ± 0.44^{b}	86.53 ± 0.74^{c}	82.67 ± 0.66^{d}

Data are presented as means±SEM (n=30).
$^{a\text{-}b}$Means with the same superscript in a row are not significantly different ($P<0.05$) from each other.

pH, acidity and TBA value were also adversely affected by the addition of even 15 ppm iron as ferrous lactate hydrate and the values were significantly different from control milk ($P<0.05$) (table 4.8), suggesting that ferrous lactate hydrate was having major effect on milk system affecting pH, acidity and oxidative stability even at 15 ppm level of addition. Sensory analysis scores of ferrous lactate hydrate fortified milk were not comparable to control milk. Rosenthal *et al*. (1993) reported that there was decrease in pH of milk samples; and pH of fortified milk kept on decreasing throughout storage of 7 days, however, magnitude of decrease in pH of ferrous lactate (5 mg/100ml) fortified milk and control milk was comparable throughout the storage duration (7 days). Ravikiran *et al.* (1977) reported that there was not huge difference in acidity of control and fortified milk with ferric lactose after 24 hrs of refrigerated storage. Increasing ferric lactose concentration upto 30 ppm concentration did not increase TBA value of milk (Ravikiran *et al.* 1977).

Table 4.8: Effect of added ferrous lactate hydrate at different concentrations on pH, acidity and TBA value of milk

Iron conc. used (ppm)	pH	Acidity (%)	TBA (at 532 nm)
Control	6.75 ± 0.010^{a}	0.133 ± 0.001^{a}	0.023 ± 0.001^{a}
15	6.74 ± 0.010^{b}	0.135 ± 0.002^{b}	0.027 ± 0.001^{b}
20	6.72 ± 0.010^{c}	0.137 ± 0.001^{c}	0.029 ± 0.001^{b}
25	6.70 ± 0.003^{d}	0.140 ± 0.001^{d}	0.036 ± 0.002^{c}

Data are presented as means±SEM (n=3).
$^{a\text{-}b}$Means with the same superscript in a column are not significantly different ($P<0.05$) from each other.

The overall sensory scores obtained for monosodium ferric EDTA fortified milk samples were 90.17, 86.27 and 78.53 for the added iron concentrations 15, 20 and 25 ppm, respectively as compared to 94.37 obtained for control milk (table 4.9). There was significant difference between the colour and appearance, odour, taste and total scores of control and fortified milk samples (at all levels of addition). The fortified milk samples above 15 ppm level of addition, showed significant difference in mouthfeel. The fortified milk samples were significantly different from control at all concentrations ($P<0.05$). Some type of foreign flavour was detected in milk upon addition of monosodium ferric EDTA which was not native to milk.

Table 4.9: Effect of added monosodium ferric EDTA at different concentrations on sensory scores of milk

Characteristics	Maximum score	Monosodium ferric EDTA			
		Iron concentration (ppm)			
		Control	**15**	**20**	**25**
Colour and appearance	10	9.5±0.02^{a}	9.17±0.05^{b}	8.93±0.07^{c}	8.37±0.1^{d}
Odour	20	18.7±0.04^{a}	17.13±0.17^{b}	16.8±0.18^{c}	15.57±0.17^{d}
Taste	40	37.6±0.11^{a}	35.77±0.21^{b}	33.87±0.27^{c}	30.77±0.54^{d}
Mouthfeel	30	28.57±0.06^{a}	28.1±0.05^{a}	26.67±0.23^{b}	23.83±0.48^{c}
Total score	100	94.37±0.22^{a}	90.17±0.49^{b}	86.27±0.83^{c}	78.53±1.34^{d}

Data are presented as means±SEM (n=30).
$^{a-b}$Means with the same superscript in a row are not significantly different ($P<0.05$) from each other.

pH of fortified milk did not get affected adversely after addition of this iron salt upto 20 ppm level of addition, however, significant difference was observed after 20 ppm level of addition ($P<0.05$). Acidity values were significantly different beyond 15 ppm and TBA value was significantly different from control milk even at 15 ppm level of addition ($P<0.05$) (table 4.10), indicating that this iron salt caused significant changes in milk system and also negatively affected the oxidative stability of milk. Sensory analysis results also showed that there was appreciable difference in scores of monosodium ferric EDTA fortified milk compared to control milk.

Table 4.10: Effect of added monosodium ferric EDTA at different concentrations on pH, acidity and TBA value of milk

Iron conc. used (ppm)	pH	Acidity (%)	TBA (at 532 nm)
Control	6.81±0.01^{a}	0.131±0.001^{a}	0.020±0.001^{a}
15	6.81±0.01^{a}	0.131±0.001^{a}	0.023±0.001^{b}
20	6.80±0.01^{ab}	0.133±0.001^{b}	0.026±0.002^{c}
25	6.79±0.01^{b}	0.136±0.001^{c}	0.031±0.002^{d}

Data are presented as means±SEM (n=3).
$^{a-b}$Means with the same superscript in a column are not significantly different ($P<0.05$) from each other.

The overall sensory scores obtained for BioFer (microencapsulated ferrous sulphate heptahydrate in liposome form) fortified milk samples were 91.12, 87.78 and 84.89 for the added iron concentrations 15, 20 and 25 ppm, respectively as compared to 94.1 obtained for control milk (table 4.11). Statistical analysis results revealed that the fortified milk samples were significantly different from control in terms of colour and appearance, odour, mouthfeel and overall sensory scores at all levels of fortification (P<0.05). Fortified milk differed from control milk in terms of taste beyond 15 ppm level. Some off-flavour was detected in fortified milk samples which could be due to ingredients of the wall material used to prepare ferrous sulphate liposomes.

Table 4.11: Effect of added Biofer (microencapsulated ferrous sulphate heptahydrate) at different concentrations on sensory scores of milk

Characteristics	Maximum score	BioFer			
		Iron concentration (ppm)			
		Control	15	20	25
Colour and appearance	10	9.64 ± 0.03^{a}	9.24 ± 0.04^{b}	8.64 ± 0.04^{c}	8.30 ± 0.04^{d}
Odour	20	18.95 ± 0.07^{a}	17.30 ± 0.07^{b}	16.20 ± 0.12^{c}	15.20 ± 0.19^{d}
Taste	40	36.23 ± 0.33^{a}	36.20 ± 0.22^{a}	34.85 ± 0.41^{b}	33.74 ± 0.57^{c}
Mouthfeel	30	29.28 ± 0.07^{a}	28.38 ± 0.09^{b}	27.50 ± 0.08^{c}	27.66 ± 0.48^{c}
Total score	100	94.10 ± 0.33^{a}	91.12 ± 0.28^{b}	87.78 ± 0.48^{c}	84.89 ± 0.81^{d}

Data are presented as means±SEM (n=30).
$^{a\text{-}b}$Means with the same superscript in a row are not significantly different (P<0.05) from each other.

pH of fortified milk did not get affected much after addition of this iron fortificant upto 20 ppm level of addition, however, significant difference was observed after 20 ppm level of addition (P<0.05). Acidity values were significantly different beyond 15 ppm and TBA value was significantly different from control milk even at 15 ppm level of addition (P<0.05) (table 4.12). This microencapsulated salt of iron also caused appreciable change in pH and acidity of milk and also affected TBA value of milk to a large extent. Sensory analysis scores of milk fortified with BioFer were very much different form control milk.

Table 4.12: Effect of added Biofer (microencapsulated ferrous sulphate heptahydrate) at different concentrations on pH, acidity and TBA value of milk

Iron conc. used (ppm)	pH	Acidity (%)	TBA (at 532 nm)
Control	6.83 ± 0.003^{a}	0.136 ± 0.0003^{a}	0.020 ± 0.003^{a}
15	6.82 ± 0.003^{a}	0.138 ± 0.001^{b}	0.025 ± 0.003^{ab}
20	6.80 ± 0.01^{b}	0.142 ± 0.001^{c}	0.030 ± 0.003^{b}
25	6.77 ± 0.01^{c}	0.146 ± 0.001^{d}	0.036 ± 0.003^{c}

Data are presented as means±SEM (n=3).
$^{a\text{-}b}$Means with the same superscript in a column are not significantly different (P<0.05) from each other.

The overall sensory scores obtained for ferric chloride hexahydrate fortified milk samples were 89.63, 85.53 and 79.5 for the added iron concentrations 15, 20 and 25 ppm, respectively as compared to 94.53 obtained for control milk (table 4.13). Statistically, the difference in all sensory parameters (colour and appearance, odour, taste, mouthfeel and total scores) was significant ($P<0.05$) at all concentrations used. There was a perception of metallic taste. The salt was having poor solubility and tended to form clumps which caused difficulty in its mixing.

Table 4.13: Effect of added ferric chloride hexahydrate at different concentrations on sensory scores of milk

Characteristics	Maximum score	Ferric chloride hexahydrate			
		Iron concentration (ppm)			
		Control	15	20	25
Colour and appearance	10	9.57±0.02^{a}	9.07±0.04^{b}	8.40±0.05^{c}	7.33±0.14^{d}
Odour	20	18.43±0.07^{a}	17.00±0.09^{b}	16.30±0.11^{c}	15.10±0.22^{d}
Taste	40	37.80±0.15^{a}	35.83±0.22^{b}	33.73±0.43^{c}	30.70±0.67^{d}
Mouthfeel	30	28.73±0.06^{a}	28.07±0.05^{b}	27.10±0.17^{c}	26.37±0.16^{d}
Total score	100	94.53±0.28^{a}	89.63±0.3^{b}	85.53±0.71^{c}	79.50±1.12^{d}

Data are presented as means±SEM (n=30).
$^{a-b}$Means with the same superscript in a row are not significantly different ($P<0.05$) from each other.

Significant difference was observed in pH, acidity and TBA value of fortified and control mlik at all levels of addition of iron salt ($P<0.05$) (table 4.14). Addition of ferric chloride hexahydrate caused noticeable changes in pH, acidity and TBA value and sensory scores were also not comparable with control milk. Gaucheron *et al.* (1997a) also found that there was reduction in pH of skim milk after addition of $FeCl_2$ and $FeCl_3$. This might be due to release of micellar bound H^+ ions.

Table 4.14: Effect of added ferric chloride hexahydrate at different concentrations on pH, acidity and TBA value of milk

Iron conc. used (ppm)	pH	Acidity (%)	TBA (at 532 nm)
Control	6.79±0.01^{a}	0.136±0.0010^{a}	0.023±0.001^{a}
15	6.77±0.01^{b}	0.139±0.0003^{b}	0.029±0.002^{b}
20	6.73±0.01^{c}	0.141±0.0010^{c}	0.034±0.002^{c}
25	6.71±0.01^{d}	0.144±0.0010^{d}	0.048±0.003^{d}

Data are presented as means±SEM (n=3).
$^{a-b}$Means with the same superscript in a column are not significantly different ($P<0.05$) from each other.

The overall sensory scores obtained for ferric ammonium citrate (brown) fortified milk samples were 89.69, 85.88 and 82.45 for the added iron concentrations 15, 20 and 25 ppm, respectively as compared to 95.92 obtained for control milk (table 4.15). Statistical analysis revealed that the fortified milk samples were significantly different ($P<0.05$) in all respects (colour and appearance, odour, taste and mouthfeel) from control milk (table 4.16). Natural colour and flavour of milk was appreciably affected after addition of this salt. Edmonson *et al*. (1971) reported that ferric ammonium citrate fortified milk developed rancid flavour.

Table 4.15: Effect of added ferric ammonium citrate (brown) at different concentrations on sensory scores of milk

Characteristics	Maximum score	Ferric ammonium citrate (brown)			
		Iron concentration (ppm)			
		Control	15	20	25
Colour and appearance	10	9.67±0.01^{a}	8.10±0.03^{b}	7.47±0.04^{c}	7.05±0.01^{d}
Odour	20	19.20±0.03^{a}	18.11±0.09^{b}	16.56±0.08^{c}	15.62±0.1^{d}
Taste	40	38.49±0.13^{a}	36.45±0.22^{b}	34.83±0.24^{c}	34.01±0.19^{d}
Mouthfeel	30	28.56±0.13^{a}	27.03±0.11^{b}	27.02±0.1^{b}	25.77±0.13^{c}
Total score	100	95.92±0.18^{a}	89.69±0.28^{b}	85.88±0.28^{c}	82.45±0.26^{d}

Data are presented as means±SEM (n=30).

$^{a-b}$Means with the same superscript in a row are not significantly different ($P<0.05$) from each other.

There was significant decrease in pH beyond 15 ppm level of addition, however, acidity and TBA value of fortified milk showed significant difference from control milk even at 15 ppm level of addition, indicating that addition of ferric ammonium citrate brown affects the milk properties significantly. Sensory analysis scores of fortified milk showed huge difference from control milk. Wang and King (1973) have reported that both the TBA evaluation and the average flavour score of the expert panel indicated that the FeAC fortified milk was stable as control milk.

Table 4.16: Effect of added ferric ammonium citrate (brown) at different concentrations on pH, acidity and TBA value of milk

Iron conc. used (ppm)	pH	Acidity (%)	TBA (at 532 nm)
Control	6.82±0.003[a]	0.135±0.0010[a]	0.021±0.001[a]
15	6.82±0.003[a]	0.136±0.0000[b]	0.030±0.001[b]
20	6.78±0.012[b]	0.138±0.0003[c]	0.037±0.001[c]
25	6.77±0.013[b]	0.140±0.0003[d]	0.044±0.002[d]

Data are presented as means±SEM (n=3).
[a-b]Means with the same superscript in a column are not significantly different (P<0.05) from each other.

The overall sensory scores obtained for ferric ammonium citrate (green) fortified milk samples were 92.14, 91.68 and 90.38 for the added iron concentrations 15, 20 and 25 ppm, respectively as compared to 96.87 obtained for control milk (table 4.17). Statistically, fortified milk samples differed significantly (P<0.05) from their control counterpart in terms of colour and appearance, odour, taste and total sensory scores. There was significant change in mouthfeel of fortified milk samples beyond 15 ppm level of addition compared to control milk (P<0.05) (table 4.18). Milk fortified with ferric ammonium citrate (green) lacked fresh flavour of control milk. Edmonson *et al.* (1971) reported that ferric ammonium citrate fortified milk developed rancid flavour.

Table 4.17: Effect of added ferric ammonium citrate (green) at different concentrations on sensory scores of milk

Characteristics	Maximum score	Ferric ammonium citrate (green)			
		Iron concentration (ppm)			
		Control	15	20	25
Colour and appearance	10	9.66±0.02[a]	9.05±0.09[b]	8.86±0.11[c]	8.64±0.12[d]
Odour	20	19.45±0.04[a]	17.36±0.25[b]	17.06±0.29[b]	16.63±0.34[c]
Taste	40	38.76±0.13[a]	37.57±0.24[b]	36.92±0.34[c]	36.26±0.41[d]
Mouthfeel	30	29.01±0.09[a]	28.47±0.11[ab]	28.25±0.13[b]	28.85±0.67[ab]
Total score	100	96.87±0.19[a]	92.14±0.51[b]	91.68±0.57[b]	90.38±1.01[c]

Data are presented as means±SEM (n=30).
[a-b]Means with the same superscript in a row are not significantly different (P<0.05) from each other.

There was significant change in pH and acidity of milk beyond 15 ppm level of addition, however, TBA value of fortified milk differed significantly from control even at 15 ppm level of addition which indicated that addition of ferric ammonium citrate (green) caused decrease in oxidative stability of milk fat. Wang and King (1973) reported that both the TBA value and the average flavour scores of FeAC fortified milk were as stable as control milk.

Table 4.18: Effect of added ferric ammonium citrate (green) at different concentrations on pH, acidity and TBA value of milk

Iron conc. used (ppm)	pH	Acidity (%)	TBA (at 532 nm)
Control	6.80±0.01^{a}	0.140±0.0010^{a}	0.020±0.002^{a}
15	6.80±0.01^{a}	0.140±0.0010^{a}	0.026±0.002^{b}
20	6.77±0.01^{b}	0.142±0.0010^{b}	0.035±0.001^{c}
25	6.76±0.01^{b}	0.145±0.0003^{c}	0.039±0.001^{d}

Data are presented as means±SEM (n=3).
$^{a-b}$Means with the same superscript in a column are not significantly different (P<0.05) from each other.

All iron salts except ferric pyrophosphate (FPP) soluble and ferrous gluconate (FG) hydrate produced distinguishable changes in colour, odour and flavour even below 15 ppm level of iron addition. However, ferric pyrophosphate (FPP) soluble and ferrous gluconate (FG) hydrate fortified milk samples gave satisfactory sensory acceptability upto 25 ppm iron. Based on the above results, the salts rejected were ferric chloride hexahydrate, ferrous lactate hydrate, monosodium ferric EDTA, ferric ammonium citrate green and brown and BioFer. Ferric ammonium citrate (brown) produced distinguishable change in colour and flavour of milk. Ferric ammonium citrate (green), monosodium ferric EDTA and ferrous lactate hydrate brought noticeable changes in flavour, the sour taste being dominant in case of ferrous lactate. Ferric chloride hexahydrate did not dissolve properly in milk and formed clumps while mixing and problem of settling down of salt at the bottom was also observed. Biofer (commercial microencapsulated ferrous sulphate) also generated some sort of off-flavour in milk. Therefore, on the basis of sensory scores, FPP soluble and FG hydrate were selected (@15, 20 and 25 ppm iron) for further study.

Our results for pH of iron fortified milk were in corroboration with Rosenthal *et al*. 1993 and Gaucheron *et al.* (1997a) who have also reported slight decrease in pH of milk of milk after addition of iron salts. Gaucheron *et al.* (1997a) reported that addition of iron ions to skim milk resulted in pH decrease. This decrease in pH could be ascribed to the acidities of iron solutions and to exchanges between iron ions added and micellar bound H^+. Ravikiran *et al.* (1977) also reported that no detrimental effect occurred on pH after addition of ferric lactose and ascorbic acid.

The present findings also showed that there was slight, however, no detrimental effect of iron addition on pH of milk. Slight increase in acidity after addition of iron to milk could be related to the decrease in pH of milk caused by iron salts addition to milk. Our results were in agreement with Ravikiran *et al.* (1977) who also reported that there was not much difference in acidity of control and fortified milk with ferric lactose after 24 hrs storage under refrigerated conditions. Wang and King (1973) reported that there was an increase in TBA value of milk when iron salts were added. Kehagias and Radhma (1973) stated that under certain circumstances, particularly in products with a low protein and high milk fat content, the presence of iron causes oxidative deterioration. However, Ravikiran *et al.* (1977) reported that increasing ferric lactose concentration upto 30 ppm concentration did not increase TBA value of milk.

4.1.2 Iron microencapsulation

Ferrous sulphate microcapsules were formed by spray cooling method with the help of droplet generator using sodium alginate/sodium alginate+apple pectin powder as wall material. The microcapsules produced a fibrous network upon freeze drying. There was problem of sedimentation of capsules at the bottom when these microcapsules were added to milk due to the difference in density of milk and capsules. Hence, these microcapsules were rejected as they formed large volume of sediments at the bottom and some microcapsules tended to stick to the walls of container, thereby decreasing the amount of iron that will be available to the consumer. Jayalalitha *et al.* (2012) used sodium alginate for iron microencapsulation and fortified yoghurt with these microcapsules. The iron fortified yoghurt had TBA value comparable to control yoghurt. However, in present investigation, alginate added to milk did not give a satisfactory product due to the poor solubility of alginate microcapsules in the liquid milk system owing to the difference in density of milk and alginate capsules.

Ferrous sulphate microcapsules were also formed by spray cooling method with the help of airless paint sprayer using polyglycerol monostearate (PGMS) as wall material. The properties of PGMS made it very difficult to be sucked and sprayed. The microcapsules formed were very light and floated on the surface of milk. Kwak *et al.* (2001) reported that PGMS solution was difficult to spray even when it was heated to 55ºC. Based on the above observations, this method of microencapsulation using PGMS was also rejected. However, Kwak *et al.* (2003) reported that there was non significant effect of addition of iron microcapsules @100 ppm iron (using PGMS as wall material) on sensory acceptability and fat oxidation of milk. They also showed a possible application of PGMS in capsulated iron fortification which may be used effectively in food systems. Abbasi and Azari (2011) also reported that milk fortified with PGMS-microencapsulated iron showed highest similarity to control particularly at low concentration (7 ppm) and PGMS was found very efficient wall material for iron microencapsulation.

Liposomes were formed by reverse phase evaporation method using soya lecithin liquid, soya lecithin powder and egg lecithin as wall materials. When liposomes of soya lecithin liquid were added to milk, these tended to clump together and floated on the surface of milk. Moreover, the typical smell of soya was pronounced in the milk in which these soya lecithin liposomes were added. Liposomes made from soya lecithin powder easily dispersed in milk system. However, problem of off-flavour was very much pronounced, which could not be masked even after addition of artificial flavour (cardamom). Liposomes formed using egg lecithin easily dispersed in milk system, however, the problem of off-flavour was still there.

It was observed that addition of artificial flavour (cardamom) masked the flavour of egg lecithin to some extent. Initially, milk was fortified with the liposome prepared by egg lecithin @30 ppm iron and flavoured with cardamom essence, however, the sensory acceptability of fortified milk was very poor. There were some oil droplets on the surface of pasteurised fortified milk upon cooling. The fortified milk differed from control milk in odour, taste and mouthfeel, suggesting that addition of ferrous sulphate liposomes brought adverse changes in odour and taste of milk and therefore, this level was rejected and further sensory analysis was conducted at lower concentrations viz. 5, 10 and 15 ppm iron.

The overall sensory scores obtained for ferrous sulphate liposomes fortified milk samples were 94.53, 91.44 and 79.85 for the added iron concentrations 5, 10 and 15 ppm, respectively as compared to 95.39 obtained for control milk (table 4.19). Sensory analysis of milk fortified with ferrous sulphate liposomes (@5, 10 and 15 ppm iron) revealed that there was significant difference ($P<0.05$) in all sensory aspects viz. colour and appearance, odour, taste and mouthfeel beyond 5 ppm level of iron addition. This indicated that liposomes could be added to milk only upto 5 ppm level in order to achieve sensory acceptability comparable to control milk. Abbasi and Azari (2011) also reported that milk fortified with liposomes showed significant difference from control in terms of sensory acceptability even at low levels (7 ppm). However, Xia and Xu (2005) reported that fluid milk fortified with ferrous sulphate liposomes (@15 ppm iron) did not differ greatly from control milk in colour and off-flavour; they suggested that ferrous sulphate liposomes may provide a powerful iron fortificant and can improve iron stability in food vehicles.

Table 4.19: Effect of added ferrous sulphate heptahydrate liposomes at different concentrations on sensory scores of milk

Characteristics	**Maximum score**	**Ferrous sulphate liposomes**			
		Iron concentration (ppm)			
		Control	**5**	**10**	**15**
Colour and appearance	10	9.47 ± 0.05^{a}	9.34 ± 0.06^{ab}	9.23 ± 0.06^{b}	8.82 ± 0.09^{c}
Odour	20	19.04 ± 0.10^{a}	18.84 ± 0.10^{a}	17.73 ± 0.13^{b}	15.06 ± 0.11^{c}
Taste	40	38.02 ± 0.27^{a}	37.62 ± 0.26^{a}	36.68 ± 0.26^{b}	32.80 ± 0.35^{c}
Mouthfeel	30	28.86 ± 0.14^{a}	28.74 ± 0.14^{a}	27.80 ± 0.16^{b}	23.17 ± 0.38^{c}
Total score	100	95.39 ± 0.44^{a}	94.53 ± 0.41^{a}	91.44 ± 0.4^{b}	79.85 ± 0.49^{c}

Data are presented as means±SEM (n=30).
$^{a-b}$Means with the same superscript in a row are not significantly different ($P<0.05$) from each other.

The photographs of ferrous sulphate liposomes prepared using egg lecithin as wall material (figure 4.1) demonstrated that liposomes had an irregular spherical shape, with a smooth surface. The diameter of liposomes was 2.4-4.82 μm (average 2.89 μm). Abbasi and Azari (2011) also reported that ferrous sulphate liposomes when observed under light microscope (1000X magnification) exhibited an irregular spherical shape.

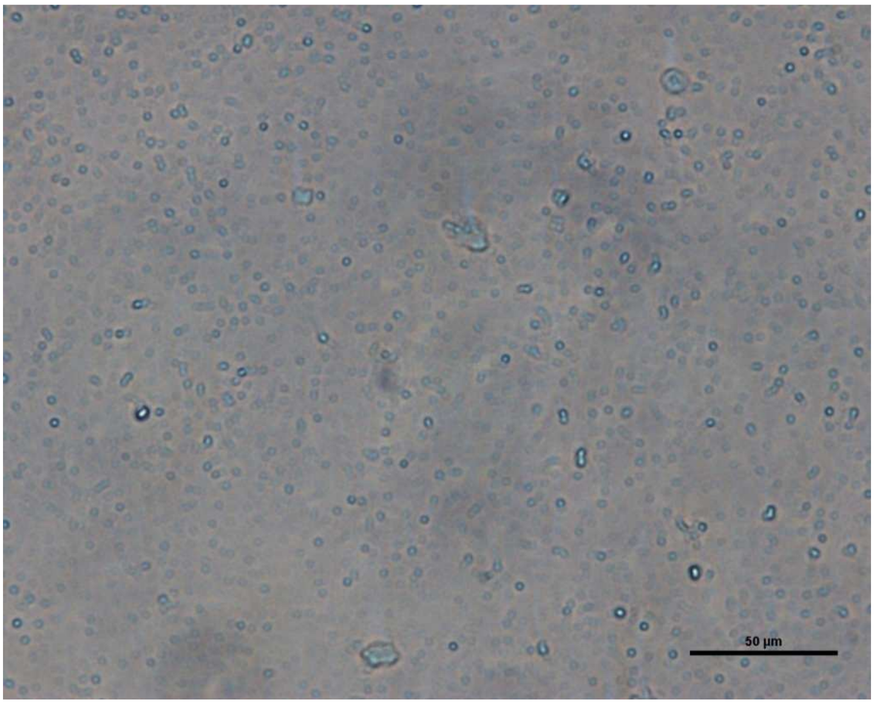

Figure 4.1: Microscopic observation of ferrous sulphate liposomes using egg lecithin as wall material (400X magnification)

Highest microencapsulation efficiency achieved was 89% (ranging from 87.5 to 89%), which was determined as per the method described by Abbasi and Azari (2011). According to Lichtenberg’s model, with increasing concentration of surfactant (Tween 80), the liposomes can partially or entirely dissolve and this process leads to partial or complete damage of liposomes and finally leakage of their contents (entrapped iron) (Abbasi and Azari 2011). However, it is noteworthy that the concentration of Tween 80 used in the present investigation did not have any detrimental effect on microencapsulation efficiency of egg lecithin liposomes. Similarly, Abbasi and Azari (2011) also reported that microencapsulation efficiency of liposomes was 85%.

Although the size of liposomes prepared using egg lecithin was small enough that these mixed in milk satisfactorily and efficiency of microencapsulation was also high (87.5-89%), however, it is evident from the results of sensory analysis that addition of ferrous sulphate liposomes caused significant changes in sensory acceptability of milk, therefore, these could be added only upto 5 ppm iron level, which is comparatively very less than the level of fortification (25 ppm) achieved by FPP soluble and FG hydrate (the unencapsulated salts of iron) without significantly affecting milk's acceptability and physico-chemical properties.

Therefore, microencapsulation experiments were not pursued further as low levels of fortification could only be achieved by this technique. Moreover, there was a pronounced effect of wall material (egg lecithin) on sensory attributes of milk. The cost of fortification also increased substantially due to the additional ingredients used in liposome preparation (wall material, solvents and stabilisers). Li (2009) also reported that the high cost of the formulated nutrients made liposomes economically viable only in high-valued food products, rather than in inexpensive staples like milk.

4.1.3 Sensory analysis scores, pH and acidity of milk fortified with vitamin A esters

The overall sensory scores obtained for vitamin A palmitate fortified milk samples were 95.03, 94.03 and 92.80 for the added concentrations 2000, 2500 and 3000 IU/L, respectively as compared to 96.3 obtained for control milk (table 4.20). Statistically, the fortified milk samples differed from their control counterparts in all sensory attributes (colour and appearance, odour, taste, mouthfeel and total scores) at all levels of addition ($P<0.05$). Some herbal smell was detected in the milk samples fortified with vitamin A palmitate.

Table 4.20: Effect of added vitamin A palmitate at different levels on sensory scores of milk

Characteristics	**Maximum score**	**Vitamin A palmitate**			
		Level (IU/L)			
		Control	**2000**	**2500**	**3000**
Colour and appearance	10	9.67 ± 0.02^a	9.67 ± 0.02^a	9.60 ± 0.02^b	9.50 ± 0.01^c
Odour	20	19.03 ± 0.04^a	18.70 ± 0.03^b	18.37 ± 0.03^c	18.10 ± 0.04^d
Taste	40	38.53 ± 0.12^a	37.93 ± 0.12^b	37.63 ± 0.12^c	36.87 ± 0.12^d
Mouthfeel	30	29.07 ± 0.05^a	28.73 ± 0.07^b	28.43 ± 0.07^c	28.30 ± 0.05^d
Total score	100	96.3 ± 0.20^a	95.03 ± 0.21^b	94.03 ± 0.2^c	92.80 ± 0.21^d

Data are presented as means±SEM (n=30).

$^{a-b}$Means with the same superscript in a row are not significantly different ($P<0.05$) from each other.

Non significant change was observed in the pH and acidity of control and fortified milk ($P<0.05$) (table 4.21), indicating that addition of vitamin A palmitate to milk did not affect physico-chemical properties of milk.

Table 4.21: Effect of added vitamin A palmitate at different levels on pH and acidity of milk

Level used (IU/L)	pH	Acidity (%)
Control	6.78 ± 0.04^a	0.136 ± 0.001^a
2000	6.78 ± 0.04^a	0.136 ± 0.001^a
2500	6.78 ± 0.04^a	0.136 ± 0.001^a
3000	6.78 ± 0.04^a	0.136 ± 0.001^a

Data are presented as means±SEM (n=3).

$^{a\text{-}b}$Means with the same superscript in a column are not significantly different ($P<0.05$) from each other.

The overall sensory scores obtained for vitamin A acetate fortified milk samples were 93.47, 93.43 and 92.12 for the added concentrations 2000, 2500 and 3000 IU/L, respectively as compared to 93.63 obtained for control milk (table 4.22). Statistical analysis revealed that the fortified milk showed non significant difference from control milk in terms of colour, appearance and mouthfeel at all levels of addition ($P<0.05$). There was noticeable change in odour and taste in the fortified sample containing 3000 IU/L of added vitamin A acetate. Total sensory scores of fortified milk altered significantly beyond 2500 IU/L level of addition. Fortified milk (upto 2500 IU/L) was similar to control milk in all aspects of sensory analysis and no change could be detected between control and fortified milk sample.

Table 4.22: Effect of added vitamin A acetate at different levels on sensory scores of milk

Characteristics	Maximum score	Vitamin A acetate			
		Level (IU/L)			
		Control	2000	2500	3000
Colour and appearance	10	9.43 ± 0.02^a	9.43 ± 0.02^a	9.43 ± 0.02^a	9.39 ± 0.02^a
Odour	20	18.43 ± 0.09^a	18.33 ± 0.11^a	18.33 ± 0.11^a	17.90 ± 0.11^b
Taste	40	37.30 ± 0.06^a	37.27 ± 0.05^a	37.08 ± 0.05^a	36.47 ± 0.05^b
Mouthfeel	30	28.47 ± 0.03^a	28.43 ± 0.04^a	28.43 ± 0.04^a	28.37 ± 0.04^a
Total score	100	93.63 ± 0.13^a	93.47 ± 0.16^a	93.43 ± 0.15^a	92.12 ± 0.11^b

Data are presented as means±SEM (n=30).

$^{a\text{-}b}$Means with the same superscript in a row are not significantly different ($P<0.05$) from each other.

Non significant change was observed in the pH and acidity of control and fortified milk ($P<0.05$) (table 4.23), indicating that addition of vitamin A acetate to milk did not affect physico-chemical properties of milk.

Table 4.23: Effect of added vitamin A acetate at different levels on pH and acidity of milk

Level used (IU/L)	pH	Acidity (%)
Control	6.75±0.02^{a}	0.138±0.001^{a}
2000	6.75±0.02^{a}	0.138±0.001^{a}
2500	6.75±0.02^{a}	0.138±0.001^{a}
3000	6.75±0.02^{a}	0.138±0.001^{a}

Data are presented as means±SEM (n=3).
$^{a-b}$Means with the same superscript in a column are not significantly different ($P<0.05$) from each other.

Vitamin A palmitate fortified milk was having some herbal smell, so was not liked by the judges. Vitamin A acetate fortified milk (@ 2000, 2500 and 3000 IU/L) was quite similar to control in terms of sensory acceptability, except the highest level (3000 IU) brought some suspicious changes in taste and mouthfeel. Therefore, vitamin A acetate (@2500 IU) was selected for milk fortification and further study. Dorai and Kahn (1990) also reported that the sensory evaluation of fortified milk with vitamin A concentrations ranging from 3000-5000 IU/L was found to be similar to that of control samples.

4.1.4 Sensory analysis scores, pH, acidity and TBA value of multiple fortified milk (vitamin A+iron)

The overall sensory scores obtained for multiple fortified milk (vitamin A acetate @2500 IU/L) with ferric pyrophosphate soluble were 96.89, 96.85 and 96.78 for the added iron concentrations 15, 20 and 25 ppm, respectively as compared to 96.92 obtained for control milk (table 4.24). Statistically, the control and fortified milk samples showed non significant difference ($P<0.05$) in terms of colour and appearance, odour, taste, mouthfeel and total scores at all levels of addition. No change was observed by the sensory panel in natural flavour and odour of milk upon fortification.

Table 4.24: Effect of added vitamin A acetate@2500 IU/L+ferric pyrophosphate soluble at different concentrations on sensory scores of milk

Characteristics	Maximum score	Vitamin A acetate@2500 IU/L+FPP soluble			
		Iron concentration (ppm)			
		Control	**15**	**20**	**25**
Colour and appearance	10	9.72±0.02^{a}	9.72±0.02^{a}	9.72±0.02^{a}	9.71±0.02^{a}
Odour	20	19.12±0.08^{a}	19.11±0.08^{a}	19.08±0.08^{a}	19.03±0.08^{a}
Taste	40	38.42±0.13^{a}	38.41±0.12^{a}	38.39±0.13^{a}	38.39±0.13^{a}
Mouthfeel	30	29.66±0.05^{a}	29.66±0.05^{a}	29.66±0.05^{a}	29.66±0.05^{a}
Total score	100	96.92±0.15^{a}	96.89±0.15^{a}	96.85±0.15^{a}	96.78±0.15^{a}

Data are presented as means±SEM (n=30).
$^{a-b}$Means with the same superscript in a row are not significantly different (P<0.05) from each other.

Slight but significant change was observed in pH, acidity and TBA value beyond 20 ppm level of addition (P<0.05) (table 4.25).

Table 4.25: Effect of added vitamin A acetate@2500 IU/L+ferric pyrophosphate soluble at different levels on pH, acidity and TBA value of milk

Iron conc. used (ppm)	pH	Acidity (%)	TBA (at 532 nm)
Control	6.86±0.01^{a}	0.139±0.001^{a}	0.020±0.001^{a}
15	6.86±0.01^{a}	0.139±0.001^{a}	0.021±0.001^{a}
20	6.85±0.01^{a}	0.139±0.001^{a}	0.022±0.001^{a}
25	6.83±0.01^{b}	0.141±0.002^{b}	0.026±0.001^{b}

Data are presented as means±SEM (n=3).
$^{a-b}$Means with the same superscript in a column are not significantly different (P<0.05) from each other.

The overall sensory scores obtained for multiple fortified milk (vitamin A acetate @2500 IU) with ferrous gluconate (FG) hydrate were 97.65, 97.25 and 97.14 for the added iron concentrations 15, 20 and 25 ppm, respectively as compared to 97.38 obtained for control milk (table 4.26). Statistically, the fortified milk samples did not differ significantly (P<0.05) in colour and appearance, odour, taste, mouthfeel and total scores from control milk at all levels of addition (P<0.05). No change was observed by the sensory panel in natural flavour and odour of milk upon fortification.

Table 4.26: Effect of added vitamin A acetate@2500 IU/L+ferrous gluconate hydrate at different concentrations on sensory scores of milk

Characteristics	Maximum score	Vitamin A acetate@2500 IU/L+FG hydrate			
		Iron concentration (ppm)			
		Control	15	20	25
Colour and appearance	10	9.74±0.02^{a}	9.74±0.02^{a}	9.74±0.02^{a}	9.72±0.02^{a}
Odour	20	19.26±0.08^{a}	19.24±0.08^{a}	19.21±0.09^{a}	19.18±0.08^{a}
Taste	40	38.87±0.11^{a}	38.85±0.11^{a}	38.83±0.11^{a}	38.8±0.11^{a}
Mouthfeel	30	29.51±0.04^{a}	29.82±0.34^{a}	29.46±0.05^{a}	29.44±0.05^{a}
Total score	100	97.38±0.16^{a}	97.65±0.39^{a}	97.25±0.17^{a}	97.14±0.16^{a}

Data are presented as means±SEM (n=30).
$^{a-b}$Means with the same superscript in a row are not significantly different (P<0.05) from each other.

Significant change in acidity and TBA value was observed beyond 20 ppm level of iron addition, however, fortified milk differed significantly in pH beyond 15 ppm level of iron addition (P<0.05) (table 4.27).

Table 4.27: Effect of added vitamin A acetate@2500 IU/L+ferrous gluconate hydrate at different levels on pH, acidity and TBA value of milk

Iron conc. used (ppm)	pH	Acidity (%)	TBA (at 532 nm)
Control	6.74±0.01^{a}	0.135±0.0003^{a}	0.023±0.001^{a}
15	6.74±0.01^{a}	0.135±0.0003^{a}	0.025±0.001^{ab}
20	6.72±0.01^{b}	0.135±0.0003^{a}	0.027±0.001^{b}
25	6.71±0.01^{b}	0.138±0.0003^{b}	0.031±0.002^{c}

Data are presented as means±SEM (n=3).
$^{a-b}$Means with the same superscript in a column are not significantly different (P<0.05) from each other.

The present findings for both types of fortified milks (VA@2500+FPP25 and VA@2500+FG25) were in agreement with findings of Edmonson *et al.* (1971); Wang and King (1973); Ravikiran *et al*. (1977); Saini *et al.* (1987) and Rosenthal *et al*. (1993). Rosenthal *et al.* (1993) reported that there was decrease in pH of milk samples, however, magnitude of decrease in pH of ferrous lactate (5 mg/100 ml) fortified milk and control milk was comparable throughout the storage duration. Gaucheron *et al.* (1997a) also found that there was reduction in pH of skim milk after addition of $FeCl_2$ and $FeCl_3$. This might be due to release of micellar bound H^+ ions. Ravikiran *et al*. (1977) also reported that there was no detrimental effect on pH after addition of ferric lactose and ascorbic acid. The present findings also showed that there was slight but no detrimental effect of iron and vitamin A addition on pH of milk apparently. Ravikiran *et al*. (1977) reported that there was not much difference in acidity of control and fortified milk with ferric lactose after 24 hrs of refrigerated storage (4-7ºC). Wang and King (1973) reported four times higher TBA values in ferrous sulphate fortified cow's milk kept for 48 hrs in a cold storage as compared to the ferric ammonium citrate fortified counterpart. Edmonson *et al*. (1971) reported that ferrous sulphate addition at 10 ppm resulted in oxidised flavour, whereas, ferric ammonium citrate containing milks developed rancid flavours, but not oxidised. Saini *et al*. (1987) observed that there were negligible differences in the TBA values of 48 and 96 hrs stored pasteurised milks fortified with 10 ppm of iron as ferrous and ferric iron with or without ascorbic acid.

Sensory analysis revealed that there was non significant difference in scores assigned to control milk and fortified milk at all levels of addition, suggesting that the milk fortified with vitamin A acetate@2500 IU/L and FPP soluble@15, 20, 25 ppm or FG hydrate@15, 20, 25 ppm showed no difference from control and was comparable to control in terms of sensory acceptability. Although, there was slight difference in pH, acidity and TBA value of control and fortified milk samples, however, the magnitude of difference was neglected considering the comparable sensory scores of control and fortified milk samples. Heat stability experiments of milk were then conducted by adding selected iron salts i.e. FPP soluble and FG hydrate (@15, 20 and 25 ppm iron).

4.2 Heat stability of control and fortified milk

4.2.1 Heat stability of control toned milk (cow milk:buffalo milk 1:1)

Heat stability as determined by the heat coagulation time/pH (HCT/pH) profile is, essentially, a measure of the temperature required to cause instantaneous coagulation (Miller and Sommer 1940). The heat stability (as heat coagulation time) of control milk (cow milk:buffalo milk 1:1) at its natural pH and after adjustment of the pH between 6.4 to 7.0 at 0.1 unit intervals using disodium hydrogen phosphate and sodium dihydrogen phosphate was studied and the results are as shown in table

4.28 and figure 4.2. The results revealed that HCT of toned milk (cow milk:buffalo milk 1:1) at 140±1°C at its natural pH (6.76) was 25.73 minutes and ranged from 31.68 minutes (at pH 6.5) to 2.41 minutes (at pH 7.0) and the maxima in the HCT/pH curve (at pH 6.5: 31.68 minutes) was on the acidic side of natural pH of milk (pH 6.66). HCT at pH 6.4 was 29.71 minutes and there was a rise in the heat coagulation time at pH 6.5 (31.68 minutes). Further increase in pH above 6.5 caused a gradual decrease in heat coagulation time upto pH 6.7 followed by a steep decline thereafter.

Table 4.28: Heat stability of control (toned mixed milk)

pH→ Samples↓	HCT (in minutes) at 140ºC							
	Milk pH	**pH adjusted with Na_2HPO_4 and NaH_2PO_4**						
	6.66 ±0.02	**6.4**	**6.5**	**6.6**	**6.7**	**6.8**	**6.9**	**7.0**
1.	26.57	29.25	31.44	29.26	24.5	3.43	3.15	2.26
2.	24.10	30.05	32.06	28.50	22.45	5.10	4.10	2.45
3.	26.51	29.82	31.54	27.15	25.57	5.15	4.18	2.54
Average	25.73 ±0.81	29.71 ±0.24	31.68 ±0.19	28.30 ±0.62	24.17 ±0.92	4.56 ±0.57	3.81 ±0.33	2.41 ±0.08

Data are presented as means±SEM (n=3).

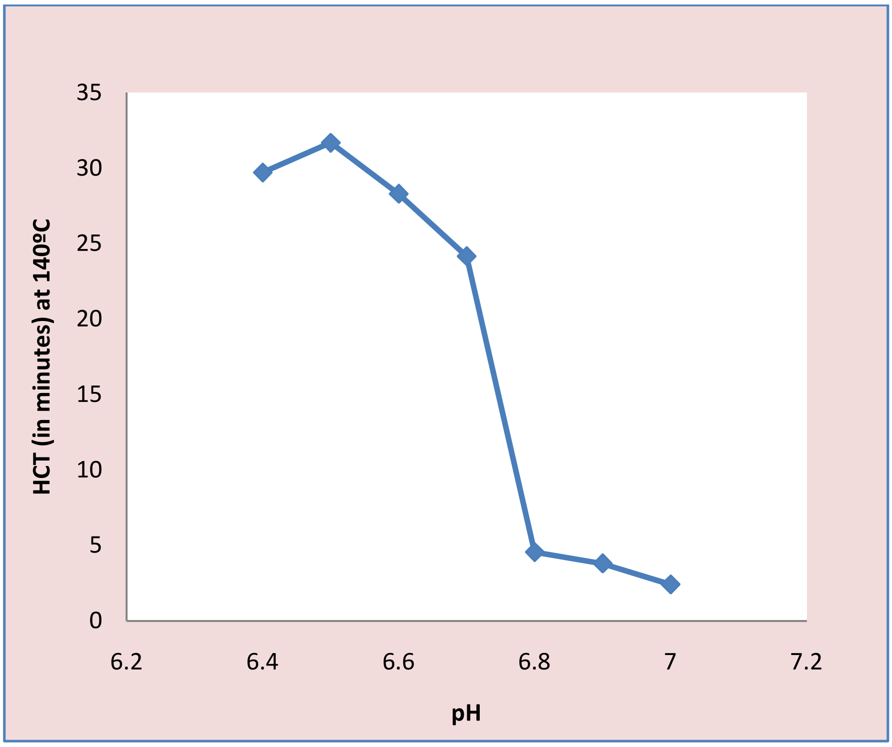

Figure 4.2: HCT/pH graph of control toned milk

4.2.2 Effect of iron and vitamin A fortification on the heat stability of toned milk (cow milk:buffalo milk 1:1)

Results regarding the effect of fortification of toned milk (cow milk:buffalo milk 1:1) with two different salts of iron (ferric pyrophosphate soluble and ferrous gluconate hydrate) which were selected on the basis of sensory scores, pH, acidity and TBA value and their three levels (15, 20 and 25 ppm) are presented in table (4.29 to 4.34) while figure (4.3 to 4.8) depict the trend of the effect of pH on the heat stability of iron fortified milk.

4.2.2.1 Heat stability of toned milk fortified with ferric pyrophosphate soluble (@15 ppm iron)

The heat stability (as heat coagulation time) of ferric pyrophosphate (FPP) soluble fortified toned milk (cow milk:buffalo milk 1:1) (@15 ppm iron) at its natural pH and after adjustment of the pH between 6.4 to 7.0 at 0.1 unit intervals using disodium hydrogen phosphate and sodium dihydrogen phosphate was studied and the results are as shown in table 4.29 and figure 4.3. The results revealed that heat coagulation time of control toned milk at 140±1°C at its natural pH (6.75) was 25.57 minutes and that of FPP soluble fortified milk (pH 6.75) was 25.78 minutes i.e. negligible difference was observed in the heat coagulation time of control milk and fortified milk at their natural pH. HCT of fortified milk ranged from 39.93 minutes (pH 6.5) to 5.94 minutes (at pH 7.0) and the maxima in HCT/pH curve (at pH 6.5: 39.93 minutes) was on the acidic side of natural pH of fortified milk (pH 6.75). HCT at pH 6.4 was 35.75 minutes and there was a rise in the heat coagulation time at pH 6.5 (39.93 minutes). Further increase in pH above 6.5 caused a gradual decrease in heat coagulation time upto pH 6.7 followed by a steep decline thereafter. Since, control and fortified milk samples were showing comparable heat coagulation times at their natural pH, therefore, it can be concluded that addition of FPP soluble@15 ppm did not affect the heat stability of milk adversely.

Table 4.29: Heat stability of FPP soluble fortified milk (@15 ppm iron)

pH→ Samples↓	**HCT (in minutes) at 140ºC**								
	Ctrl milk pH	**Fortified milk (FPP soluble@15 ppm iron)**							
		Fortd milk pH	**pH adjusted with Na_2HPO_4 and NaH_2PO_4**						
	6.75 ±0.04	**6.75 ±0.05**	**6.4**	**6.5**	**6.6**	**6.7**	**6.8**	**6.9**	**7.0**
1.	23.47	23.98	36.71	41.23	37.84	23.86	8.91	7.52	6.47
2.	26.87	26.94	35.39	39.87	38.22	23.64	7.16	6.74	5.39
3.	26.37	26.42	35.16	38.69	37.42	21.28	7.38	6.41	5.97
Average	25.57 ±0.75	25.78 ±0.65	35.75 ±0.48	39.93 ±0.73	37.83 ±0.23	22.93 ±0.83	7.82 ±0.55	6.89 ±0.33	5.94 ±0.31

Data are presented as means±SEM (n=3).

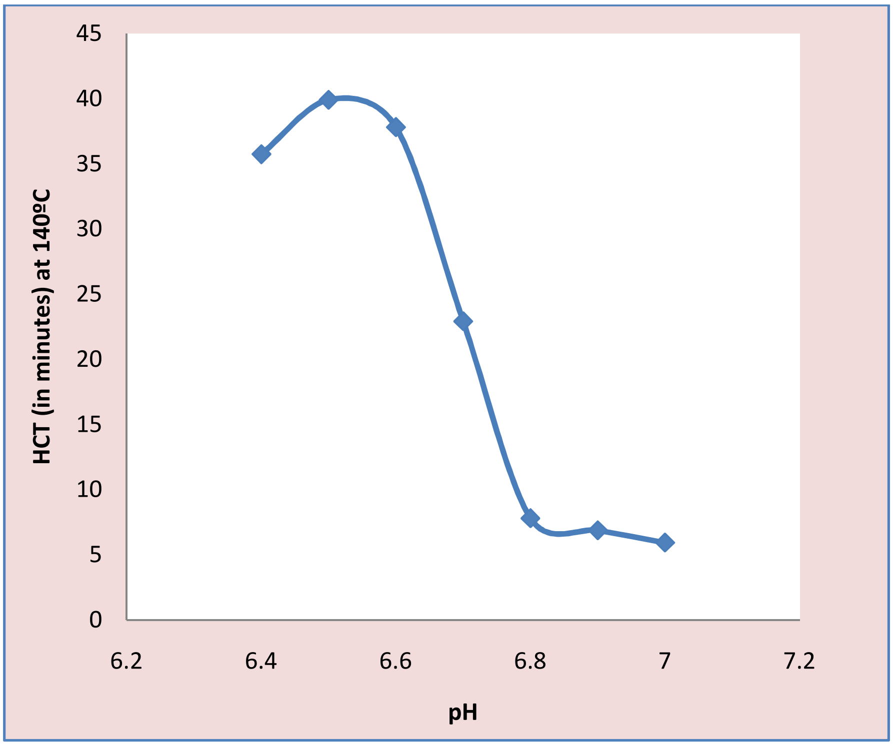

Figure 4.3: HCT/pH graph of milk fortified with FPP soluble (@15 ppm iron)

4.2.2.2 Heat stability of toned milk fortified with ferric pyrophosphate soluble (@20 ppm iron)

The heat stability (as heat coagulation time) of ferric pyrophosphate (FPP) soluble fortified toned milk (cow milk:buffalo milk 1:1) (@20 ppm iron) at its natural pH and after adjustment of the pH between 6.4 to 7.0 at 0.1 unit intervals using disodium hydrogen phosphate and sodium dihydrogen phosphate was studied and the results are as shown in table 4.30 and figure 4.4. The results revealed that heat coagulation time of control toned milk at 140±1°C at its natural pH (6.75) was 22.64 minutes and that of FPP soluble fortified milk (pH 6.73) was 22.59 minutes i.e. negligible difference was observed in the heat coagulation time of control milk and fortified milk at their natural pH. HCT of fortified milk ranged from 37.57 minutes (pH 6.5) to 5.16 minutes (at pH 7.0) and the maxima in HCT/pH curve (at pH 6.5: 37.57 minutes) was on the acidic side of natural pH of fortified milk (pH 6.73). HCT at pH 6.4 was 33.95 minutes and there was a rise in the heat coagulation time at pH 6.5 (37.57 minutes). Further increase in pH above 6.5 caused a gradual decrease in heat coagulation time upto pH 6.7 followed by a steep decline thereafter. Since, control and fortified milk samples were showing comparable heat coagulation times at their natural pH, therefore, it can be concluded that increasing the level of addition FPP soluble upto 20 ppm did not affect the heat stability of milk adversely.

Table 4.30: Heat stability of FPP soluble fortified milk (@20 ppm iron)

	HCT (in minutes) at 140ºC								
		Fortified milk (FPP soluble@20 ppm iron)							
	Ctrl milk pH	Fortd milk pH	pH adjusted with Na_2HPO_4 and NaH_2PO_4						
pH→ Samples↓	**6.75 ±0.04**	**6.73 ±0.04**	**6.4**	**6.5**	**6.6**	**6.7**	**6.8**	**6.9**	**7.0**
1.	22.25	22.48	35.06	39.15	36.10	21.25	7.35	6.50	6.00
2.	21.45	21.25	32.24	35.38	35.20	20.50	5.25	5.00	4.40
3.	24.23	24.06	34.56	38.18	37.18	23.68	6.57	7.03	5.09
Average	22.64 ±0.83	22.59 ±0.81	33.95 ±0.87	37.57 ±1.13	36.16 ±0.57	21.81 ±0.96	6.39 ±0.61	6.18 ±0.61	5.16 ±0.46

Data are presented as means±SEM (n=3).

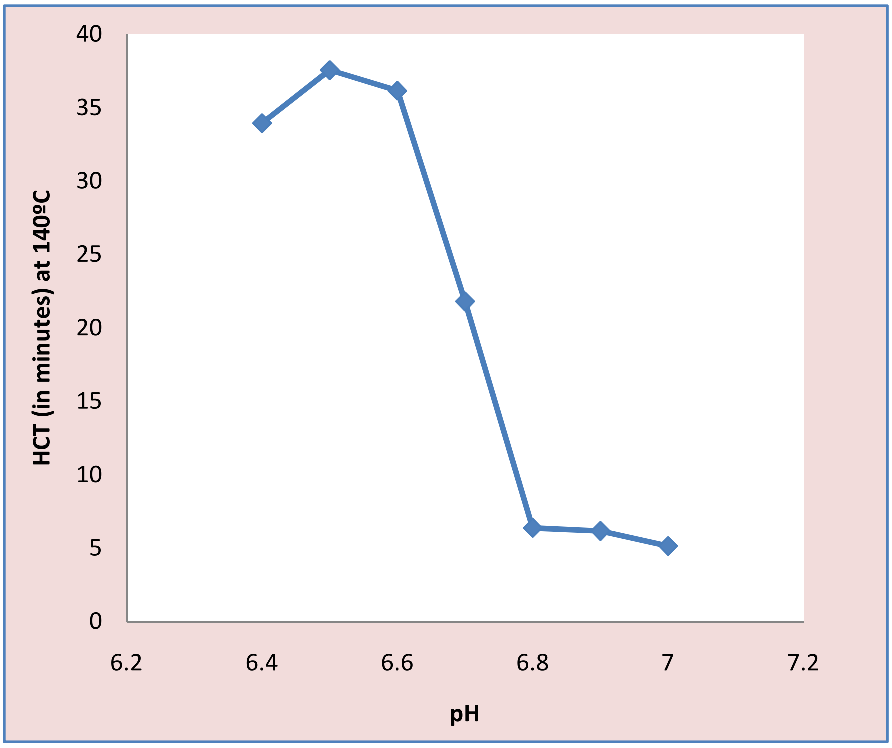

Figure 4.4: HCT/pH graph of milk fortified with FPP soluble (@20 ppm iron)

4.2.2.3 Heat stability of toned milk fortified with ferric pyrophosphate soluble (@25 ppm iron)

The heat stability (as heat coagulation time) of ferric pyrophosphate (FPP) soluble fortified toned milk (cow milk:buffalo milk 1:1) (@25 ppm iron) at its natural pH and after adjustment of the pH between 6.4 to 7.0 at 0.1 unit intervals using disodium hydrogen phosphate and sodium dihydrogen phosphate was studied and the results are as shown in table 4.31 and figure 4.5. The results revealed that heat coagulation time of control toned milk at 140±1°C at its natural pH (6.68) was 27.09 minutes and that of FPP soluble fortified milk (pH 6.66) was 26.64 minutes i.e. negligible difference was observed in the heat coagulation time of control milk and fortified milk at their natural pH. HCT of fortified milk ranged from 35.98 minutes (pH 6.5) to 3.05 minutes (at pH 7.0) and the maxima in HCT/pH curve (at pH 6.5: 35.98 minutes) was on the acidic side of natural/unadjusted pH of fortified milk (pH 6.66). HCT at pH 6.4 was 32.54 minutes and there was a rise in the heat coagulation time at pH 6.5 (35.98 minutes). Further increase in pH above 6.5 caused a gradual decrease in heat coagulation time upto pH 6.7 followed by a steep decline thereafter. Since, control and fortified milk samples were showing comparable heat coagulation times at their natural pH, therefore, it can be concluded that FPP soluble can be added to milk upto 25ppm without significantly affecting its heat stability.

Table 4.31: Heat stability of FPP soluble fortified milk (@25 ppm iron)

pH→ Samples↓	HCT (in minutes) at 140ºC								
	Ctrl milk pH	Fortified milk (FPP soluble@25 ppm iron)							
		Fortd milk pH	pH adjusted with Na_2HPO_4 and NaH_2PO_4						
	6.68 ±0.01	6.66 ±0.01	6.4	6.5	6.6	6.7	6.8	6.9	7.0
1.	30.40	30.05	30.40	34.54	34.04	27.48	5.30	4.10	3.35
2.	22.50	22.40	33.09	36.78	36.27	28.53	3.45	2.45	2.30
3.	28.36	27.48	34.13	36.47	35.87	26.94	4.30	4.15	3.50
Average	27.09 ±2.37	26.64 ±2.25	32.54 ±1.11	35.98 ±0.70	35.39 ±0.68	27.65 ±0.47	4.35 ±0.53	3.57 ±0.56	3.05 ±0.38

Data are presented as means±SEM (n=3).

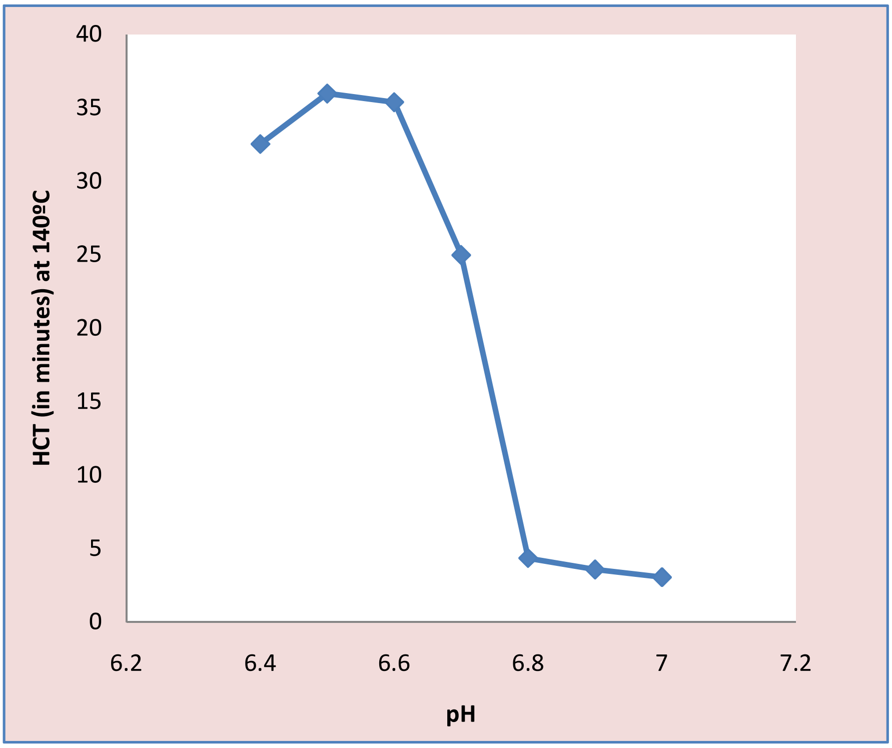

Figure 4.5: HCT/pH graph of milk fortified with FPP soluble (@25 ppm iron)

4.2.2.4 Heat stability of toned milk fortified with ferrous gluconate hydrate (@15 ppm iron)

The heat stability (as heat coagulation time) of ferrous gluconate (FG) hydrate fortified toned milk (cow milk:buffalo milk 1:1) (@15 ppm iron) at its natural pH and after adjustment of the pH between 6.4 to 7.0 at 0.1 unit intervals using disodium hydrogen phosphate and sodium dihydrogen phosphate was studied and the results are as shown in table 4.32 and figure 4.6. The results revealed that heat coagulation time of control toned milk at 140±1°C at its natural pH (6.72) was 31.93 minutes and that of FG hydrate fortified milk (pH 6.70) was 31.41 minutes i.e. negligible difference was observed in the heat coagulation time of control milk and fortified milk at their natural pH. HCT of fortified milk ranged from 39.27 minutes (pH 6.5) to 6.13 minutes (at pH 7.0) and the maxima in HCT/pH curve (at pH 6.5: 39.27 minutes) was on the acidic side of natural pH of fortified milk (pH 6.70). HCT at pH 6.4 was 33.8 minutes and there was a rise in the heat coagulation time at pH 6.5 (39.27 minutes). Further increase in pH above 6.5 caused a gradual decrease in heat coagulation time upto pH 6.7 followed by a steep decline thereafter. Since, control and fortified milk samples were showing comparable heat coagulation times at their natural pH, therefore, it can be concluded that addition of FG hydrate@15 ppm did not affect the heat stability of milk negatively.

Table 4.32: Heat stability of FG hydrate fortified milk (@15 ppm iron)

	HCT (in minutes) at 140°C								
		Fortified milk (FG hydrate@15 ppm iron)							
	Ctrl milk pH	**Fortd milk pH**	**pH adjusted with Na_2HPO_4 and NaH_2PO_4**						
pH→ Samples↓	**6.72 ±0.02**	**6.70 ±0.02**	**6.4**	**6.5**	**6.6**	**6.7**	**6.8**	**6.9**	**7.0**
1.	30.28	30.08	31.25	37.86	35.29	28.67	7.58	6.27	5.68
2.	33.25	32.48	34.78	38.67	36.82	29.68	8.57	7.26	6.22
3.	32.26	31.68	35.36	41.28	39.69	25.67	8.36	7.14	6.49
Average	31.93 ±0.87	31.41 ±0.71	33.8 ±1.28	39.27 ±1.03	37.27 ±1.29	28.01 ±1.20	8.17 ±0.30	6.89 ±0.31	6.13 ±0.24

Data are presented as means±SEM (n=3).

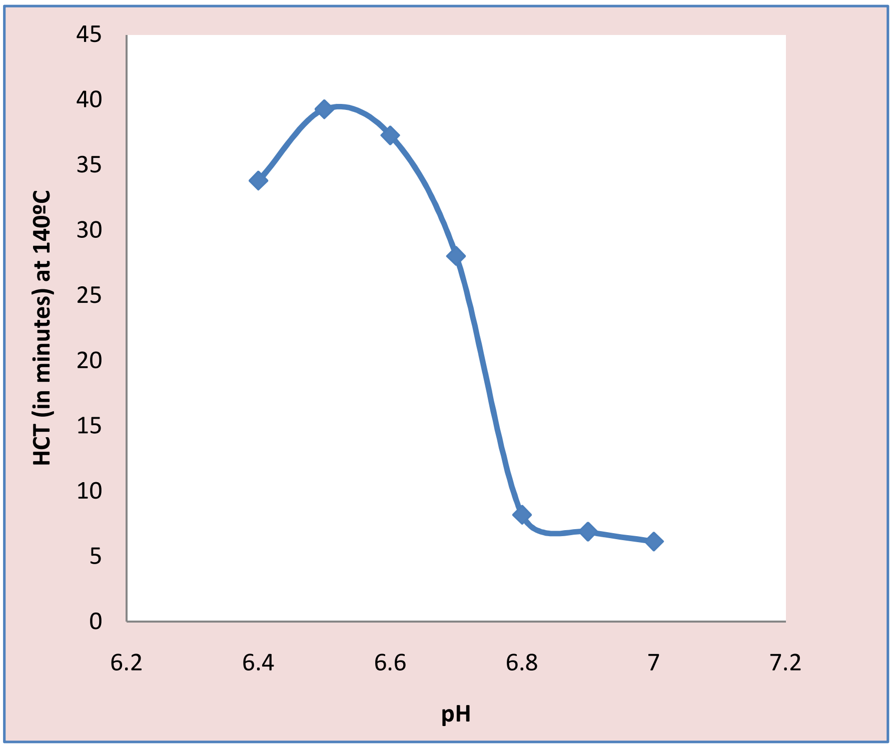

Figure 4.6: HCT/pH graph of milk fortified with FG hydrate (@15 ppm iron)

4.2.2.5 Heat stability of toned milk fortified with ferrous gluconate hydrate (@20 ppm iron)

The heat stability (as heat coagulation time) of ferrous gluconate (FG) hydrate fortified toned milk (cow milk:buffalo milk 1:1) (@20 ppm iron) at its natural pH and after adjustment of the pH between 6.4 to 7.0 at 0.1 unit intervals using disodium hydrogen phosphate and sodium dihydrogen phosphate was studied and the results are as shown in table 4.33 and figure 4.7. The results revealed that heat coagulation time of control toned milk at 140±1°C at its natural pH (6.75) was 26.67 minutes and that of FG hydrate fortified milk (pH 6.73) was 26.48 minutes i.e. negligible difference was observed in the heat coagulation time of control milk and fortified milk at their natural pH. HCT of fortified milk ranged from 37.22 minutes (pH 6.5) to 5.28 minutes (at pH 7.0) and the maxima in HCT/pH curve (at pH 6.5: 37.22 minutes) was on the acidic side of natural pH of fortified milk (pH 6.73). HCT at pH 6.4 was 32.58 minutes and there was a rise in the heat coagulation time at pH 6.5 (37.22 minutes). Further increase in pH above 6.5 caused a gradual decrease in heat coagulation time upto pH 6.7 followed by a steep decline thereafter. Since, control and fortified milk samples were showing comparable heat coagulation times at their natural pH, therefore, it can be concluded that increasing the concentration of FG hydrate upto 20 ppm did not affect the heat stability of milk negatively.

Table 4.33: Heat stability of FG hydrate fortified milk (@20 ppm iron)

	HCT (in minutes) at 140ºC								
		Fortified milk (FG hydrate@20 ppm iron)							
	Ctrl milk pH	**Fortd milk pH**	**pH adjusted with Na_2HPO_4 and NaH_2PO_4**						
pH→ Samples↓	**6.75 ±0.03**	**6.73 ±0.03**	**6.4**	**6.5**	**6.6**	**6.7**	**6.8**	**6.9**	**7.0**
1.	26.58	26.06	32.06	36.08	35.94	24.38	6.54	6.30	6.15
2.	28.11	28.34	30.54	35.37	33.46	27.19	6.13	5.58	5.32
3.	25.32	25.04	35.13	40.22	39.11	23.29	7.22	6.23	6.00
Average	26.67 ±0.81	26.48 ±0.98	32.58 ±1.35	37.22 ±1.51	36.17 ±1.64	24.95 ±1.16	6.63 ±0.32	6.04 ±0.23	5.28 ±0.26

Data are presented as means±SEM (n=3).

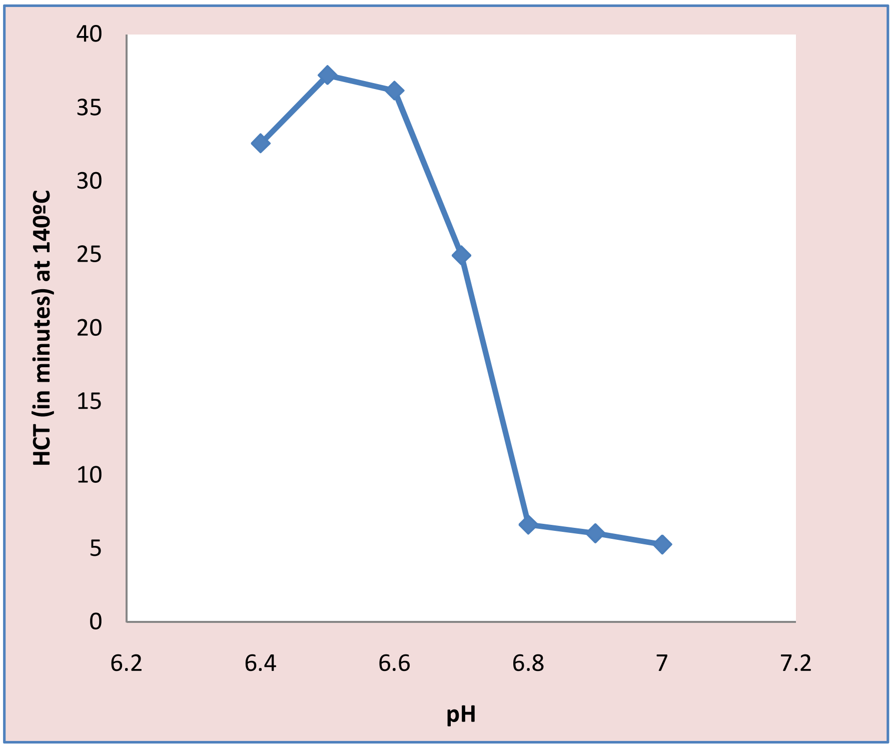

Figure 4.7: HCT/pH graph of milk fortified with FG hydrate (@20 ppm iron)

4.2.2.6 Heat stability of milk fortified with ferrous gluconate hydrate (@25 ppm iron)

The heat stability (heat coagulation time) of ferrous gluconate (FG) hydrate fortified toned milk (cow milk:buffalo milk 1:1) (@25 ppm iron) at its natural pH and after adjustment of the pH between 6.4 to 7.0 at 0.1 unit intervals using disodium hydrogen phosphate and sodium dihydrogen phosphate was studied and the results are as shown in table 4.34 and figure 4.8. The results revealed that heat coagulation time of control toned milk at 140±1°C at its natural pH (6.76) was 23.29 minutes and that of FG hydrate fortified milk (pH 6.74) was 23.62 minutes i.e. negligible difference was observed in the heat coagulation time of control milk and fortified milk at their natural pH. HCT of fortified milk ranged from 35.34 minutes (pH 6.5) to 3.1 minutes (at pH 7.0) and the maxima in HCT/pH curve (at pH 6.5: 35.34 minutes) was on the acidic side of natural pH of fortified milk (pH 6.74). HCT at pH 6.4 was 31.57 minutes and there was a rise in the heat coagulation time at pH 6.5 (35.34 minutes). Further increase in pH above 6.5 caused a gradual decrease in heat coagulation time upto pH 6.7 followed by a steep decline thereafter. It can be concluded from the above results that control and fortified milk samples were showing comparable heat coagulation times at their natural pH, therefore, it can be concluded that FG hydrate can be added to milk upto 25 ppm level without significantly affecting heat stability of milk.

Table 4.34: Heat stability of FG hydrate fortified milk (@25 ppm iron)

	HCT (in minutes) at 140ºC								
		Fortified milk (FG hydrate@25 ppm iron)							
	Ctrl milk pH	Fortd milk pH	pH adjusted with Na_2HPO_4 and NaH_2PO_4						
pH→ Samples↓	**6.76 ±0.02**	**6.74 ±0.02**	**6.4**	**6.5**	**6.6**	**6.7**	**6.8**	**6.9**	**7.0**
1.	23.38	23.06	31.36	34.18	36.08	21.47	5.94	3.48	2.58
2.	22.32	22.58	33.19	38.33	36.08	20.41	7.26	7.00	5.35
3.	24.18	25.23	30.15	33.51	32.02	21.18	5.41	5.04	2.27
Average	23.29 ±0.54	23.62 ±0.82	31.57 ±0.88	35.34 ±1.51	33.58 ±1.26	21.01 ±0.32	4.00 ±1.00	3.24 ±0.26	3.10 ±0.09

Data are presented as means±SEM (n=3).

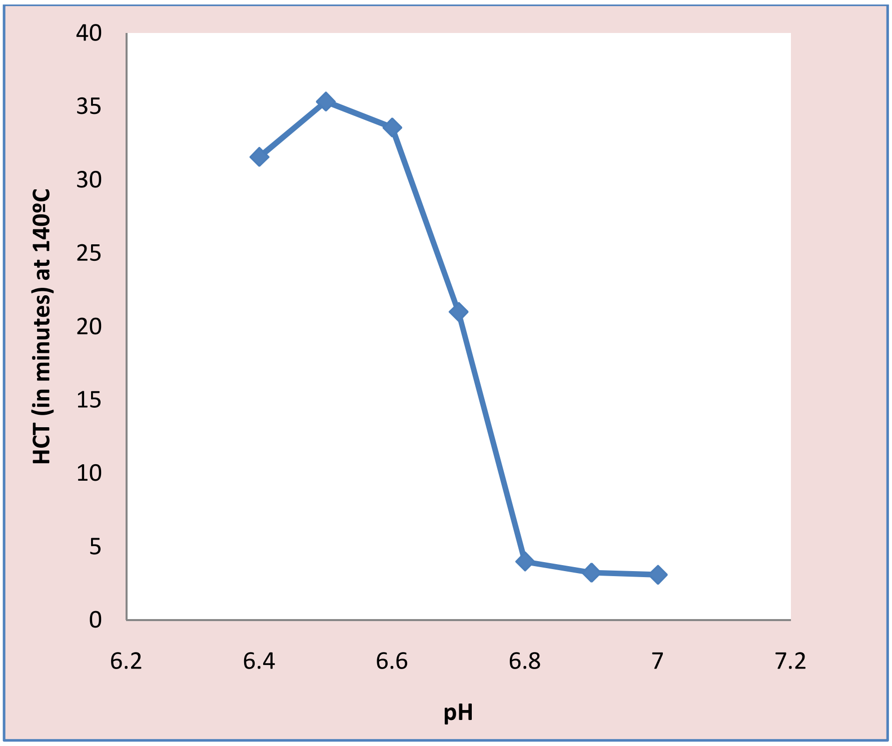

Figure 4.8: HCT/pH graph of milk fortified with FG hydrate (@25 ppm iron)

The results revealed that heat coagulation time of control, FPP soluble and FG hydrate fortified milk samples were comparable, suggesting that addition of FPP soluble and FG hydrate upto 25 ppm level of addition did not affect the heat stability of milk, thereby indicating the feasibility of development of iron fortified milk (@25 ppm iron) having heat stability comparable to control milk. A lot of work has been reported on the effect of calcium fortification on heat stability of milk at 130-140ºC (Sweetsur and Muir 1980; Tateo *et al*. 1997; Sindhu 1988; Vyas and Tong 2004; Singh *et al*. 2007). However, there are no studies available in literature on the effect of iron addition on heat stability of milk. Our results, however, were correlated with the work of Gaucheron *et al.* (1997a) who reported that heat treatments (95ºC/15 or 30 minutes) of iron supplemented skim milk samples (containing 1.5 mM of iron) induced no visible heat instability i.e. no coagulation or aggregation was observed.

It is clear from the results of present investigation that heat coagulation time of fortified milk was comparable to control milk, and therefore, no stabilisers were used for enhancing heat stability of fortified milk. The use of stabilisers in fortified milk e.g. calcium fortified cow milk, buffalo milk and soy milk has been necessitated by the drop in pH caused by the addition of fortificant salts. This drop in pH ultimately led to the decreased heat stability of fortified milk (Evenhuis 1957; Pyne 1962; Puri *et al*. 1979; Arora *et al*. 2005; Ranjan *et al*. 2005; Pathomrungsiyounggul *et al*. 2007). Sweetsur and Muir (1980) observed that the primary effect of stabilisers (salts) is a consequence of their effect on pH of milk. The comparable heat stability of control and fortified milk could be due to negligible change in pH caused by addition of iron salts at selected concentrations. Therefore, it can be concluded from the above results that fortification with either FPP soluble or FG hydrate @25 ppm iron level did not cause any adverse change in milk system which could render the milk unsuitable for heat processing treatments, i.e. iron fortified milk (@25 ppm iron) could be processed at very high temperatures without any visible coagulation or aggregation.

4.3 Effect of different processing conditions on stability of added nutrients to milk

The various steps involved in processing and storage necessary for efficient distribution and utilisation have a measurable impact on some specific nutrients. Some form of heat treatment is used in preparing practically all milk products for use by consumers. Typically this ranges from mild heat treatment like pasteurisation (63ºC/30 minutes) to sterilisation at 121ºC/15 minutes at 15 psi. Additional cooling, freezing or heating exposures normally occur, varying widely depending on the specific milk product, the food application in which it is used, the duration of the storage and distribution cycle, and the method of food preparation (Graham 1973). Generally, most nutrients in the milk system are remarkably stable under these varieties of exposures. However, some nutrients undergo significant losses due to the aforesaid conditions. Storage temperature, light exposure and availability of oxygen appear to be of major importance for vitamin stability in milk.

4.3.1 Effect of processing conditions on stability of vitamin A in milk

Toned milk was fortified with vitamin A and iron singly and in combination and subjected to different heat treatments viz. pasteurisation (63ºC/15 minutes), boiling and sterilisation (121ºC/15 minutes/15 psi); light exposure treatment (1485, 2970 and 4455 lux/0, 2, 4, 8, 16 and 32 hrs) and storage under refrigerated conditions (4-7ºC) in polyethylene pouches and glass bottles. Effect of these processing conditions on vitamin A retention in control milk (M_1), FPP soluble@25 ppm fortified milk (M_2), FG hydrate@25 ppm fortified milk (M_3), vitamin A acetate@2500 IU/L fortified milk (M_4), vitamin A acetate@2500 IU/L+FPP soluble@25 ppm fortified milk (M_5) and vitamin A acetate@2500 IU/L+FG hydrate@25 ppm fortified milk (M_6) was then evaluated.

4.3.1.1 Effect of heat treatments on vitamin A retention in milk

Vitamin A content in milk was affected severely with the increase in severity of heat treatment. The losses were in the order:

Sterilisation > Boiling > Pasteurisation

It is evident from the table 4.35 that vitamin A content of pasteurised milk samples (both control and fortified) did not significantly differ ($P<0.05$) from raw milk samples (vitamin A content of raw milk was 324.71, 332.14, 336.46, 2837.90, 2808.92 and 2809.16 IU/L for M_1, M_2, M_3, M_4, M_5 and M_6, respectively; that of pasteurised milk was 318.11, 317.17, 317.287, 2778.74, 2798.82 and 2778.32 IU/L for M_1, M_2, M_3, M_4, M_5 and M_6, respectively), suggesting that there were negligible losses of vitamin A upon pasteurisation of milk. M_2 and M_3 samples differed significantly ($P<0.05$) from rest of the samples in terms of vitamin A loss during pasteurisation (figure 4.10), however, the extent of loss was negligible suggesting that fortification with iron or vitamin A did not have any significant effect on vitamin A loss occurring during pasteurisation.

Boiling caused significant reduction in vitamin A content of both control and fortified milk samples. Vitamin A content of boiled milk samples was significantly different ($P<0.05$) from that of raw and pasteurised milk samples (vitamin A content of boiled milk was 250.21, 193.72, 179.61, 2265.54, 1940.88 and 1818.21 IU/L for M_1, M_2, M_3, M_4, M_5 and M_6, respectively) as shown in table 4.35. There was significant difference ($P<0.05$) for all the boiled milk samples and their pasteurised counterparts in terms of vitamin A loss (figure 4.9). All milk samples differed significantly ($P<0.05$) from one another in terms of vitamin A loss (figure 4.10).

There was significant difference ($P<0.05$) in vitamin A content of milk samples after sterilisation compared to their raw and pasteurised counterparts. However, there was non significant difference among sterilised milk samples and their boiled counterparts except M_1 and M_6 which also differed significantly from the respective boiled samples in terms of vitamin A content (vitamin A content of sterilised milk was 205.65, 162.39, 147.37, 2261.13, 1906.30 and 1580.92 IU/L for M_1, M_2, M_3, M_4, M_5 and M_6, respectively) as shown in table 4.35 ($P<0.05$). Significant loss ($P<0.05$) of vitamin A occurred after sterilisation of milk compared to pasteurisation and boiling for both control and fortified milk samples (figure 4.9). There was significant difference ($P<0.05$) among all the sterilised milk samples in terms of vitamin A losses (% loss was 36.866, 51.47, 134.73, 20.27, 32.15 and 77.86 for M_1, M_2, M_3, M_4, M_5 and M_6, respectively) as shown in figure 4.10.

Loss of vitamin A was higher during all heat treatments for iron fortified milk compared to milk without any added iron. This suggests that iron might have played role in accelerating the degradation of vitamin A. Wang and King (1973) reported that iron is known to catalyse the oxidation of milk lipids resulting in the development of an unpleasant odour and flavour (oxidised flavour) and may also cause loss of nutrients such as vitamin E, vitamin A and carotene. Milk undergoing oxidation also suffered from loss of the abovesaid nutrients.

In case of FG hydrate fortified milk (M_3 and M_6), the losses were more as compared to FPP soluble fortified milk (M_2 and M_5) for all heat treatments viz. pasteurisation, boiling and sterilisation (figure 4.10). This may be due to the fact that FPP soluble is a chelated salt of iron and may interact lesser with the matrix material compared to FG hydrate which is a highly soluble salt of iron and can interact and affect the milk components more than FPP soluble. Wang and King (1973) reported that the stability of the ferric ammonium citrate (FeAC) fortified milks must at least partially concern the structure of the iron salt itself. FeAC remains largely undissociated or is readily complexed to some component in milk thus inhibiting its lipid oxidative activity. They also reported that the structure of iron salt itself, in part at least, determines its potency in affecting lipid oxidation. Ferrous iron has been shown to be more detrimental than ferric iron causing oxidative deterioration (Mulder *et al.* 1949; Demott 1971; Edmonson *et al.* 1971). It has been demonstrated by Arrington and Krienke (1954) and King and Dunkley (1959) that metal chelators added to milk inhibit metal induced lipid oxidation. Similarly, ferrous ion in FG hydrate seemed to cause more oxidation, and hence greater loss of vitamin A than ferric ion (chelated with sodium citrate) in FPP soluble.

In vitamin A fortified milk (M_4), % losses during boiling and sterilisation were less compared to those occurring in control milk (M_1), indicating that added vitamin A (encapsulated) was more stable than the inherent vitamin A of milk (figure 4.10). In case of milk fortified with both vitamin A and iron salt, the losses were more than vitamin A fortified milk. However, the losses were lesser compared to milk fortified only with iron. This strengthened our belief that added (microencapsulated) vitamin A was more stable than vitamin A native to milk.

Thompson and Erdody (1974) reported that vitamin A added to milk is more susceptible to light induced degradation than the natural form present in milk. Added vitamin A is known to be destroyed more rapidly by light than its naturally occurring counterpart. Type and composition of carrier fat used in the vitamin fortification mixture and the presence of antioxidant and emulsifiers can also affect vitamin A photostability (Berge *et al*. 1987; Bartholomew and Ogden 1990; Zahar *et al.*1986, 1987, 1992). Bartholomew and Ogden (1990) **s**tudied the comparative stability of naturally occurring vitamin A in milk and that of added externally. They found that vitamin A added to milk is more light sensitive and degraded more rapidly than indigenous vitamin A when concentrated oil solutions of vitamin A and emulsifier were used for fortification.

However, it is to be pointed out that vitamin A used in the present study was in microencapsulated form (cold water soluble form so that it could be dissolved in milk easily) and greater stability of added vitamin A compared to inherent vitamin A could be ascribed to the protection of encapsulated vitamin A which might have been provided by the surrounding wall material. This wall material shielded the core material (vitamin A acetate) from the effect of processing conditions.

Our results were in accordance with Bector and Rani (1998) who observed that vitamin A content did not change appreciably when milk was pasteurised. Kumar (1973) reported 8.4% loss of vitamin A in toned milk during pasteurisation. Maguer and Jackson (1983) assessed the content and stability of vitamin A in fortified milks and reported that destruction of the vitamin A (both naturally present in milk and externally added) by pasteurisation (85ºC/15 seconds) or direct ultra-high temperature (144ºC/3-4 seconds) treatment was minimal. With pasteurisation, no difference was observed in stability of the vitamin naturally present in milk (retinyl esters and alcohol) and the synthetic form (retinyl palmitate) added during the fortification process. Bector and Rani (1998) reported a decline of 16-18% in concentration of vitamin A during boiling and sterilisation of milk. Greater losses of vitamin A during boiling and sterilization observed in the present study could be related to the addition of iron salts which might have played role in accelerating degradation of vitamin A.

Table 4.35: Effect of heat treatment on vitamin A retention in milk

Treatment→ Sample↓	**Vitamin A content in milk (IU/L)**			
	Raw milk	**Pasteurised milk**	**Boiled milk**	**Sterilised milk**
CTRL (M_1)	324.71±19.16^{a}	318.11±17.47^{a}	250.21±16.21^{b}	205.65±17.86^{c}
CTRL+FPP25 (M_2)	332.14±24.34^{a}	317.17±25.87^{a}	193.72±24.94^{b}	162.39±19.85^{b}
CTRL+FG25 (M_3)	336.46±23.71^{a}	317.287±24.82^{ab}	179.61±23.49^{b}	147.37±23.23^{b}
CTRL+VA2500 (M_4)	2837.90±40.13^{a}	2778.74±12.81^{a}	2265.54±32.02^{b}	2261.13±31.98^{b}
CTRL+VA2500 +FPP25 (M_5)	2808.92±21.97^{a}	2798.82±22.39^{a}	1940.88±30.42^{b}	1906.30±39.23^{b}
CTRL+VA2500 +FG25 (M_6)	2809.16±16.78^{a}	2778.32±19.47^{a}	1818.21±36.11^{b}	1580.92±37.02^{c}

Data are presented as means±SEM (n=3).

$^{a-b}$Means with the same superscript in a row are not significantly different (P<0.05) from each other.

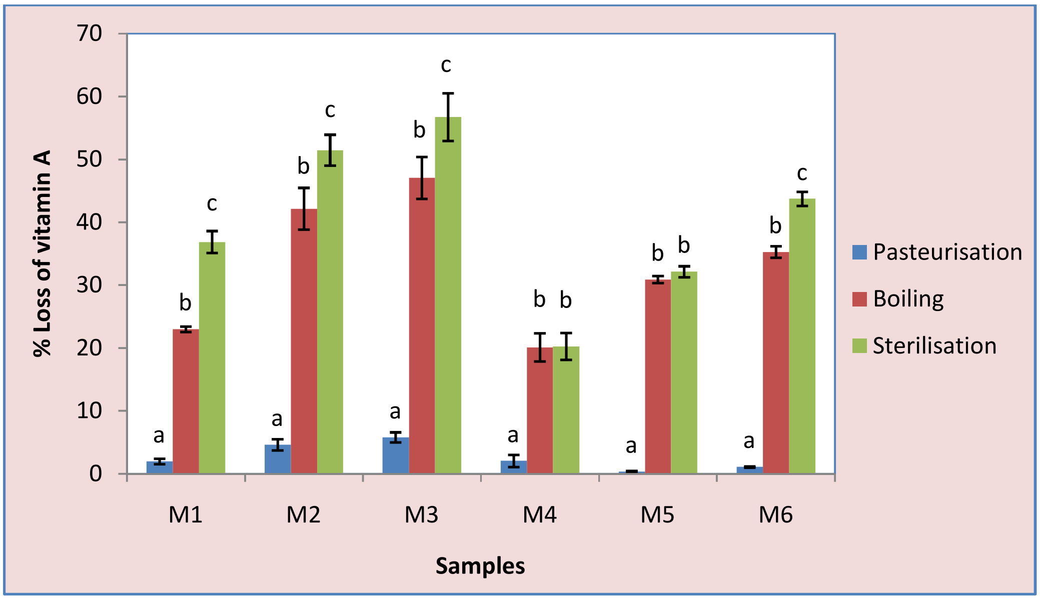

Figure 4.9: Effect of heat treatment on vitamin A retention in milk (% loss)

$^{a-b}$Samples represented with different letters are significantly different (P<0.05) from each other.

Error bars show the variations of three determinations in terms of standard error of mean.

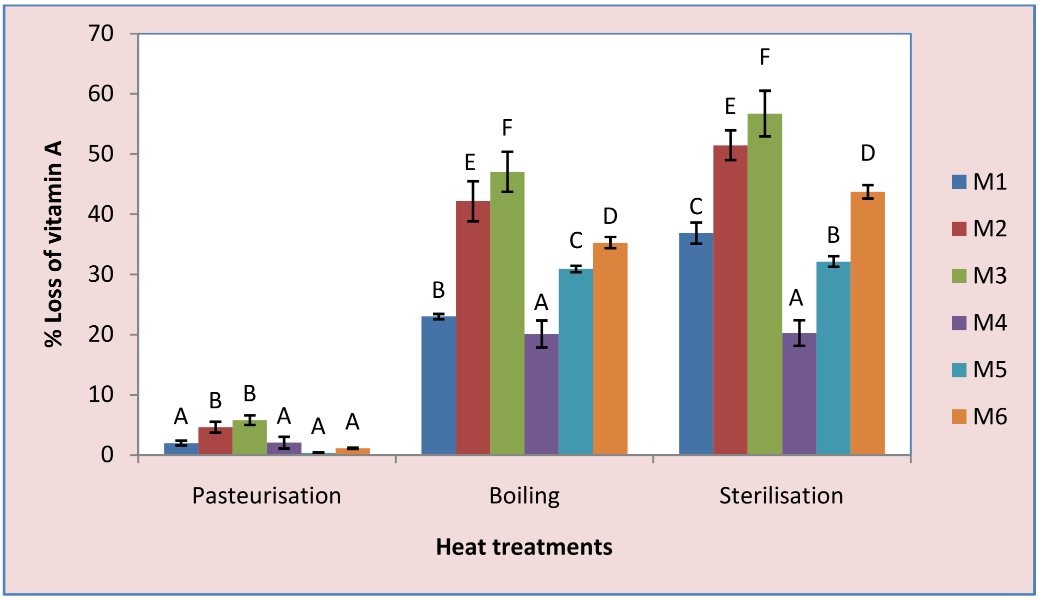

Figure 4.10: Effect of fortification on vitamin A retention in milk during different heat treatments (% loss)

$^{A\text{-}B}$Samples represented with different letters are significantly different ($P<0.05$) from each other.

Error bars show the variations of three determinations in terms of standard error of mean.

4.3.1.2 Effect of light intensity and duration of light exposure on vitamin A retention in milk

Light is known to have a damaging effect on several foodstuffs like milk and other dairy products. The sensitivity of milk and dairy products to light is attributed to the presence of light sensitive vitamins like vitamins A and B_2 and the action of vitamin B_2 as photo-sensitiser, which is able to induce a cascade of oxidative reactions leading to significant losses in other vitamins (e.g. vitamin A and D) and changes in the sensory quality (Sattar *et al*. 1977; Fanelli *et al.* 1985; Li and Min 1998). Sattar and deMan (1973) reported that the typical light intensities in dairy display cases ranged from about 1614 lux (150 ft-c) to greater than 5380 lux (500 ft-c). Therefore, milk samples were kept under refrigerated conditions (4-7ºC) and exposed to light of varying intensities (1485, 2970 and 4455 lux) for 0, 2, 4, 8, 16 and 32 hrs; and their vitamin A content was analysed to determine stability of vitamin A towards light.

4.3.1.2.1 Effect of light (1485 lux) on vitamin A retention in milk

Vitamin A content (table 4.36) decreased significantly ($P<0.05$) with increase in duration of exposure to fluorescent light (intensity 1485 lux) in all types of milk samples (vitamin A content at 0 hr was 505.96, 503.68, 506.43, 2977.52, 2944.54 and 2944.54 IU/L for M_1, M_2, M_3, M_4, M_5 and M_6, respectively; after 2 hrs was 503.56, 496.757, 495.13, 2951.45, 2881.22 and 2868.98 IU/L for M_1, M_2, M_3, M_4, M_5 and M_6, respectively; after 4 hrs was 494.85, 453.39, 444.28, 2872.91, 2706.77 and 2664.513 IU/L for M_1, M_2, M_3, M_4, M_5 and M_6, respectively; and after 32 hrs was 250.41, 156.55, 121.39, 1941.06, 1617.09 and 1508.46 IU/L for M_1, M_2, M_3, M_4, M_5 and M_6, respectively). Vitamin A content of milk samples were significantly different ($P<0.05$) after 4 hrs exposure to light, however, non significant changes in vitamin A content of milk samples were observed after 2 hrs exposure.

There was significant difference ($P<0.05$) on exposure to light for 2 hrs, 4 hrs, 8 hrs, 16 hrs and 32 hrs in all types of milk samples (% loss after 2 hrs was 0.47, 1.38, 2.5757, 0.88, 2.16 and 2.57 for M_1, M_2, M_3, M_4, M_5 and M_6, respectively; after 4 hrs was 11.03, 21.39, 23.54, 9.68, 22.54 and 25.29 for M_1, M_2, M_3, M_4, M_5 and M_6, respectively; and after 32 hrs duration was 50.54, 68.75, 76.22, 34.82, 45.08 and 48.77 for M_1, M_2, M_3, M_4, M_5 and M_6, respectively) as shown in figure 4.11.

It is evident from figure 4.12 that after 2 hrs exposure to light, there was non significant difference ($P<0.05$) in M_1, M_2 and M_4 in terms of vitamin A losses. M_2, M_5 and M_6 also did not show any significant difference in vitamin A losses, however, M_3 showed highest losses in vitamin A and differed significantly from M_1 and M_4, but not from M_2, M_5 and M_6. After 4 hrs exposure to light, non significant difference ($P<0.05$) was observed in terms of vitamin A loss between M_1 and M_4, however, these differed significantly from other milk samples. M_5 and M_6; M_2 and M_6 also showed non significant difference from each other in terms of vitamin A losses. M_3 showed highest loss of vitamin A and differed significantly from all other milk samples. After 32 hrs exposure to light of 1485 lux intensity, there was significant difference ($P<0.05$) among all the milk samples in terms of vitamin A losses (figure 4.12). The order of losses observed in milk samples after 32 hrs light exposure was:

$$M_3>M_2>M_1>M_6>M_5>M_4$$

The results indicated that there was a greater loss of vitamin A in case of FPP soluble fortified milk and FG hydrate fortified milk by the same treatment compared with that occurring in control milk. Thus, indicating that iron played a significant role and accelerated the degradation of vitamin A. In vitamin A fortified milk (M_4), % losses after 32 hours (34.82%) were much less compared to those occurring in control milk (M_1) (50.54%), indicating that added vitamin A (encapsulated) was more stable than the inherent vitamin A present in milk. In case of milk fortified with both vitamin A and iron salt, the losses were more than vitamin A fortified milk. However, the losses were less compared to milk fortified only with iron. This strengthened our belief that added (microencapsulated) vitamin A was more stable than vitamin A native to milk.

Table 4.36: Effect of light (1485 lux) on vitamin A retention in milk

Duration of exposure→ Sample↓	**Vitamin A content in milk (IU/L)**					
	0 hr	**2 hrs**	**4 hrs**	**8 hrs**	**16 hrs**	**32 hrs**
CTRL (M_1)	505.96 ±24.31^{a}	503.56 ±24.05^{a}	494.85 ±23.73^{a}	450.47 ±23.37^{b}	387.16 ±17.86^{c}	250.41 ±14.29^{d}
CTRL+FPP25 (M_2)	503.68 ±23.83^{a}	496.76 ±24.01^{a}	453.39 ±9.19^{b}	393.78 ±8.91^{c}	319.20 ±5.89^{d}	156.55 ±6.20^{e}
CTRL+FG25 (M_3)	506.43 ±24.23^{a}	495.13 ±22.75^{a}	444.28 ±23.18^{b}	387.64 ±23.20^{c}	312.49 ±20.84^{d}	121.39 ±15.92^{e}
CTRL+VA2500 (M_4)	2977.52 ±27.84^{a}	2951.45 ±30.03^{a}	2872.91 ±18.79^{b}	2689.50 ±45.14^{c}	2366.70 ±50.81^{d}	1941.06 ±48.68^{e}
CTRL+VA2500 +FPP25 (M_5)	2944.54 ±54.89^{a}	2881.22 ±57.52^{a}	2706.77 ±36.66^{b}	2280.03 ±32.01^{c}	1993.96 ±17.07^{d}	1617.09 ±41.58^{e}
CTRL+VA2500 +FG25 (M_6)	2944.54 ±54.89^{a}	2868.98 ±53.25^{a}	2664.51 ±48.89^{b}	2198.98 ±36.22^{c}	1893.50 ±15.46^{d}	1508.46 ±43.40^{e}

Data are presented as means±SEM (n=3).

$^{a-b}$Means with the same superscript in a row are not significantly different ($P<0.05$) from each other.

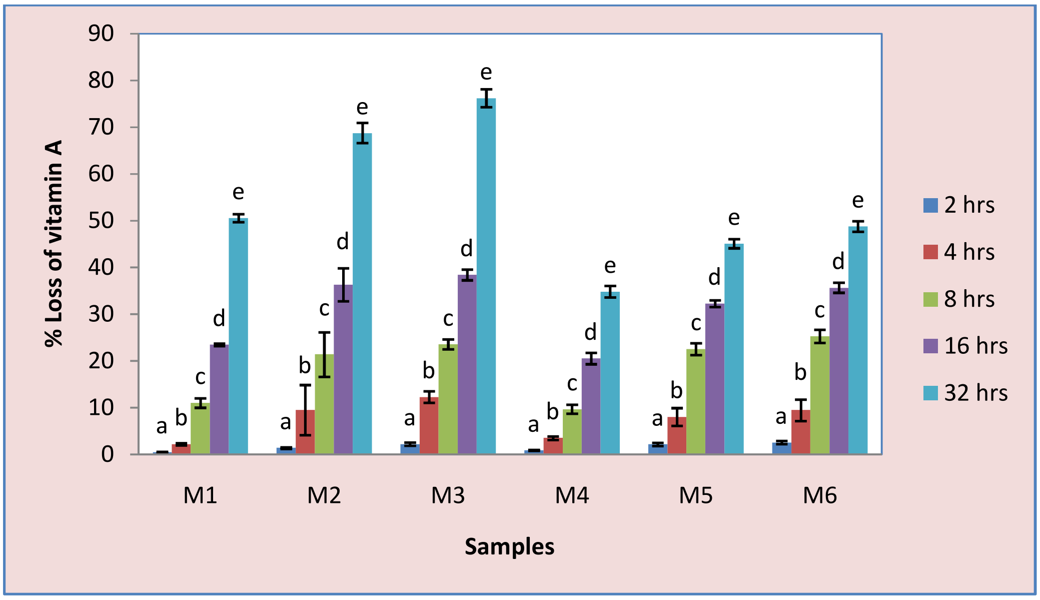

Figure 4.11: Effect of light (1485 lux) on vitamin A retention in milk (% loss)

$^{a-b}$Samples represented with different letters are significantly different (P<0.05) from each other.

Error bars show the variations of three determinations in terms of standard error of mean.

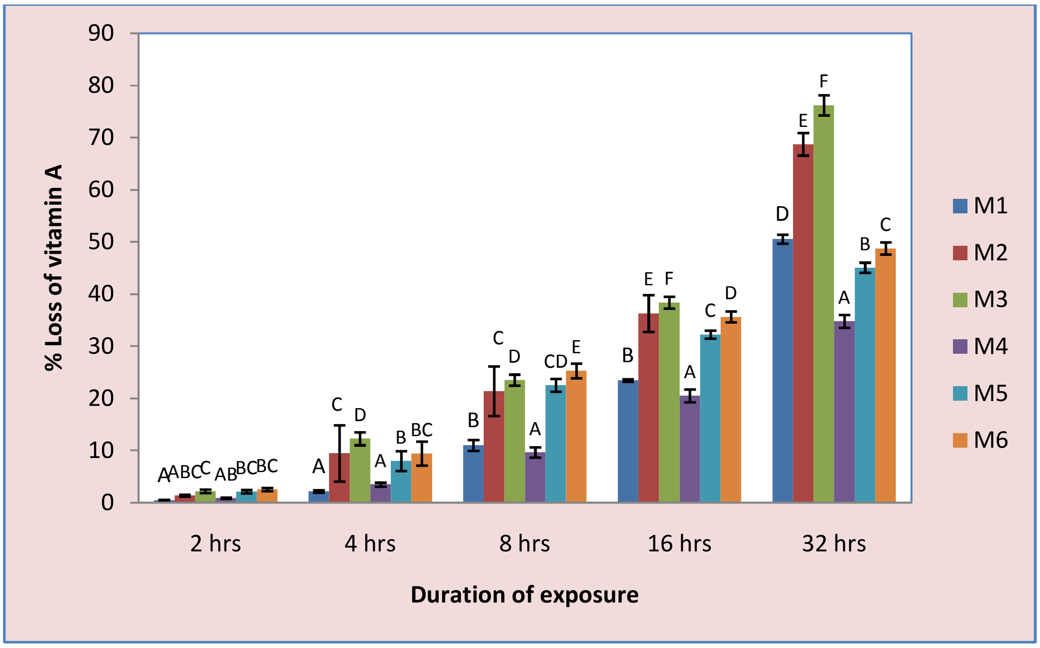

Figure 4.12: Effect of fortification on vitamin A retention in milk exposed to light (1485 lux) (% loss)

$^{A-B}$Samples represented with different letters are significantly different (P<0.05) from each other.
Error bars show the variations of three determinations in terms of standard error of mean.

4.3.1.2.2 Effect of light (2970 lux) on vitamin A retention in milk

Vitamin A degradation (table 4.37) increased significantly ($P<0.05$) with increase in duration of exposure to fluorescent light (intensity 2970 lux) in all types of milk samples (vitamin A content at 0 hr was 675.98, 671.71, 670.73, 3164.15, 3166.05 and 3163.43 IU/L for M_1, M_2, M_3, M_4, M_5 and M_6, respectively; after 2 hrs was 647.36, 614.31, 603.68, 3067.70, 3036.69 and 2959.35 IU/L for M_1, M_2, M_3, M_4, M_5 and M_6, respectively; after 4 hrs was 588.670, 561.89, 543.00, 2921.90, 2880.00 and 2819.54 IU/L for M_1, M_2, M_3, M_4, M_5 and M_6, respectively; and after 32 hrs was 171.05, 138.38, 114.65, 114.65, 1925.71, 1127.48 and 1067.40 IU/L for M_1, M_2, M_3, M_4, M_5 and M_6, respectively). Vitamin A content of milk samples was significantly different after 2 hrs exposure to light except for M_1.

It is evident from the figure 4.13 that there was significant difference ($P<0.05$) on exposure to light for 2 hrs, 4 hrs, 8 hrs, 16 hrs and 32 hrs in all types of milk samples (% loss after 2 hrs was 4.24, 8.55, 10.00, 3.04, 4.09 and 6.47 for M_1, M_2, M_3, M_4, M_5 and M_6, respectively; after 4 hrs was 12.93, 16.35, 19.06, 7.66±0.95, 9.04 and 10.88 for M_1, M_2, M_3, M_4, M_5 and M_6, respectively; and after 32 hrs duration was 74.66, 79.47, 82.92, 39.15, 64.41 and 66.28 for M_1, M_2, M_3, M_4, M_5 and M_6, respectively).

After 2 hrs exposure to light, there was non significant difference ($P<0.05$) in M_1, M_4 and M_5 in terms of vitamin A losses as shown in figure 4.14. All other milk samples differed significantly from each other in terms of vitamin A losses. After 4 hrs exposure, all milk samples showed significant difference from each other. The order of losses observed in milk samples after 32 hrs light exposure was:

$$M_3>M_2>M_1>M_6>M_5>M_4$$

The results indicated that there was greater loss of vitamin A in case of FPP soluble fortified milk and FG hydrate fortified milk by the same treatment compared with that occurring in control milk. Hence, it can be concluded that iron played a significant role in accelerating the degradation of vitamin A. In vitamin A fortified milk (M_4), % loss after 32 hours (39.15%) was much less compared to that occurring in control milk (M_1) (74.66%) as shown in figure 4.14, indicating that added vitamin A (encapsulated) was more stable than the inherent vitamin A present in milk. In case of milk fortified with both vitamin A and iron salt, the losses were more than vitamin A fortified milk. However, the losses were lesser compared to milk fortified with iron alone. This strengthened our belief that added (microencapsulated) vitamin A was more stable than vitamin A native to milk.

Table 4.37: Effect of light (2970 lux) on vitamin A retention in milk

Duration of exposure→ Sample↓	**Vitamin A content in milk (IU/L)**					
	0 hr	**2 hrs**	**4 hrs**	**8 hrs**	**16 hrs**	**32 hrs**
CTRL (M_1)	675.98 $\pm 12.92^a$	647.36 $\pm 13.79^a$	588.70 $\pm 14.84^b$	483.78 $\pm 11.96^c$	303.56 $\pm 35.65^d$	171.05 $\pm 19.84^e$
CTRL+FPP25 (M_2)	671.71 $\pm 12.24^a$	614.31 $\pm 11.34^b$	561.83 $\pm 10.34^c$	429.09 $\pm 4.51^d$	312.22 $\pm 14.09^e$	138.38 $\pm 16.26^f$
CTRL+FG25 (M_3)	670.73 $\pm 11.19^a$	603.68 $\pm 10.83^b$	543.00 $\pm 11.80^c$	392.12 $\pm 7.26^d$	260.77 $\pm 9.68^e$	114.65 $\pm 5.39^f$
CTRL+VA2500 (M_4)	3164.15 $\pm 25.62^a$	3067.70 $\pm 44.44^b$	2921.90 $\pm 44.85^c$	2613.60 $\pm 30.77^d$	2267.67 $\pm 32.99^e$	1925.71 $\pm 35.69^f$
CTRL+VA2500 +FPP25 (M_5)	3166.05 $\pm 25.99^a$	3036.69 $\pm 43.69^b$	2880.00 $\pm 40.15^c$	2356.23 $\pm 52.55^d$	1967.88 $\pm 44.62^e$	1127.48 $\pm 42.41^f$
CTRL+VA2500 +FG25 (M_6)	3163.43 $\pm 24.76^a$	2959.35 $\pm 54.48^b$	2819.54 $\pm 42.14^c$	2226.08 $\pm 75.78^d$	1852.65 $\pm 38.03^e$	1067.40 $\pm 49.65^f$

Data are presented as means±SEM (n=3).

$^{a-b}$Means with the same superscript in a row are not significantly different (P<0.05) from each other.

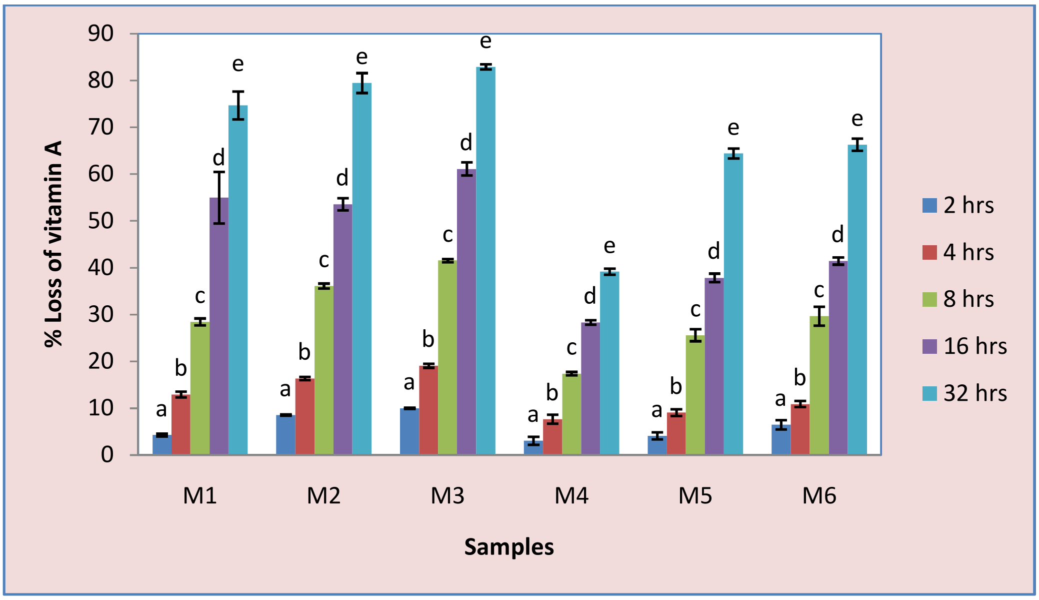

Figure 4.13: Effect of light (2970 lux) on vitamin A retention in milk (% loss)

$^{a\text{-}b}$Samples represented with different letters are significantly different ($P<0.05$) from each other.

Error bars show the variations of three determinations in terms of standard error of mean.

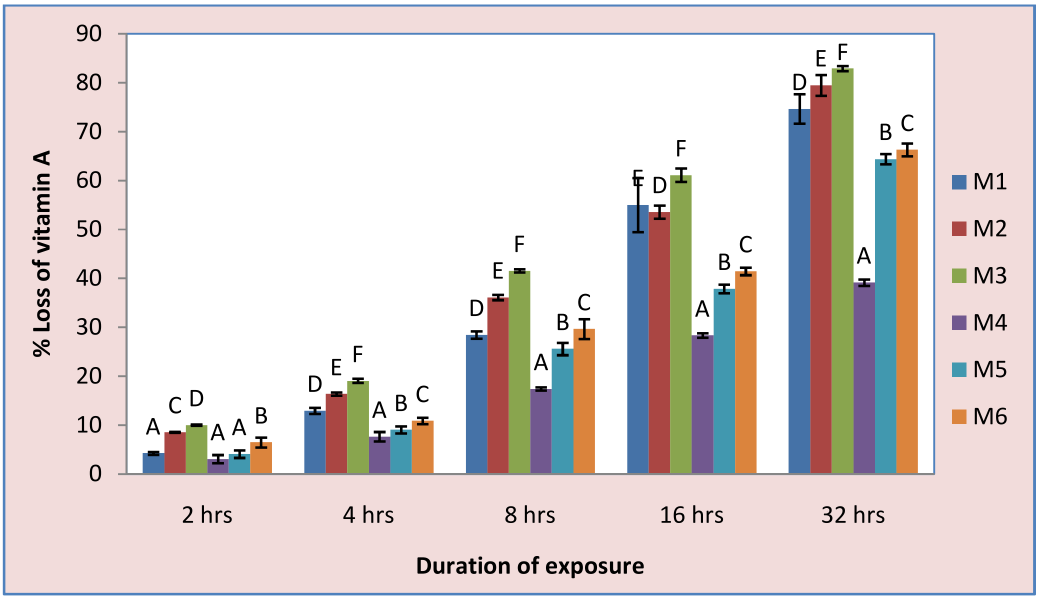

Figure 4.14: Effect of fortification on vitamin A retention in milk exposed to light (2970 lux) (% loss)

$^{A\text{-}B}$Samples represented with different letters are significantly different ($P<0.05$) from each other.

Error bars show the variations of three determinations in terms of standard error of mean.

4.3.1.2.3 Effect of light (4455 lux) on vitamin A retention in milk

Vitamin A degradation (table 4.38) increased significantly ($P<0.05$) with increase in duration of exposure to fluorescent light (intensity 4455 lux) in all types of milk samples (vitamin A content at 0 hr was 701.62, 702.32, 703.07, 3178.33, 3178.72 and 3188.11 IU/L for M_1, M_2, M_3, M_4, M_5 and M_6, respectively; after 2 hrs was 645.92, 617.04, 590.21, 2973.29, 2781.44 and 2681.55 IU/L for M_1, M_2, M_3, M_4, M_5 and M_6, respectively; after 4 hrs was 548.26, 489.87, 441.40, 2534.53, 2479.68 and 2248.05 IU/L for M_1, M_2, M_3, M_4, M_5 and M_6, respectively; and after 32 hrs was 141.15, 102.12, 52.51, 1475.57, 685.04 and 451.25 IU/L for M_1, M_2, M_3, M_4, M_5 and M_6, respectively). Vitamin A content of milk samples was significantly different after 2 hrs exposure to light except for M_1.

There was significant difference ($P<0.05$) for all types of milk samples exposed to light for 2 hrs, 4 hrs, 8 hrs, 16 hrs and 32 hrs (% loss after 2 hrs was 8.03, 12.23, 16.20, 6.46, 12.49 and 15.89 for M_1, M_2, M_3, M_4, M_5 and M_6, respectively; after 4 hrs was 12.93, 16.35, 19.06, 7.66, 9.04 and 10.88 for M_1, M_2, M_3, M_4, M_5 and M_6, respectively; and after 32 hrs duration was 79.86, 85.45, 92.57, 53.60, 78.46 and 85.87 for M_1, M_2, M_3, M_4, M_5 and M_6, respectively) as shown in figure 4.15.

After 2 hrs exposure to light, there was non significant difference in M_2 and M_5 in terms of vitamin A losses. All other milk samples differed significantly ($P<0.05$) from each other in terms of vitamin A losses (figure 4.16). The order of losses observed in milk samples after 32 hrs light exposure was:

$$M_3>M_6>M_2>M_1>M_5>M_4$$

The results indicate that there was greater loss of vitamin A in case of FPP soluble fortified milk and FG hydrate fortified milk by the same treatment compared with that occurring in control milk as discussed earlier in section 4.3.1.2.2. Thus, indicating that iron played a significant role and accelerate the degradation of vitamin A. In vitamin A fortified milk (M_4), % losses after 32 hours (53.60%) were much less compared to those occurring in control milk (M_1) (79.86%) as shown in figure 4.16, indicating that added vitamin A (encapsulated) was more stable than the inherent vitamin A present in milk. In case of milk fortified with both vitamin A and iron salt, the losses were more than vitamin A fortified milk. However, the losses were lesser compared to milk fortified only with iron.

Table 4.38: Effect of light (4455 lux) on vitamin A retention in milk

Duration of exposure→ Sample↓	**Vitamin A content in milk (IU/L)**					
	0 hr	**2 hrs**	**4 hrs**	**8 hrs**	**16 hrs**	**32 hrs**
CTRL (M_1)	701.62 ±33.40^{a}	645.92 ±37.51^{a}	548.26 ±38.29^{b}	393.00 ±29.88^{c}	209.02 ±13.66^{d}	141.15 ±5.13^{e}
CTRL+FPP25 (M_2)	702.32 ±33.69^{a}	617.04 ±38.66^{b}	489.87 ±36.71^{c}	321.50 ±31.04^{d}	141.87 ±7.30^{e}	102.12 ±4.54^{f}
CTRL+FG25 (M_3)	703.07 ±33.18^{a}	590.21 ±41.56^{b}	441.40 ±43.00^{c}	266.00 ±29.19^{d}	104.86 ±3.81^{e}	52.51 ±6.36^{f}
CTRL+VA2500 (M_4)	3178.33 ±42.77^{a}	2973.29 ±50.98^{b}	2534.53 ±53.68^{c}	2177.76 ±57.10^{d}	1756.05 ±58.73^{e}	1475.57 ±60.55^{f}
CTRL+VA2500 +FPP25 (M_5)	3178.72 ±41.69^{a}	2781.44 ±38.14^{b}	2479.68 ±32.95^{c}	2038.88 ±23.62^{d}	1431.88 ±38.27^{e}	685.04 ±21.42^{f}
CTRL+VA2500 +FG25 (M_6)	3188.11 ±41.14^{a}	2681.55 ±75.60^{b}	2248.05 ±35.93^{c}	1716.12 ±40.19^{d}	1047.83 ±40.90^{e}	451.25 ±34.90^{f}

Data are presented as means±SEM (n=3).
$^{a-b}$Means with the same superscript in a row are not significantly different ($P<0.05$) from each other.

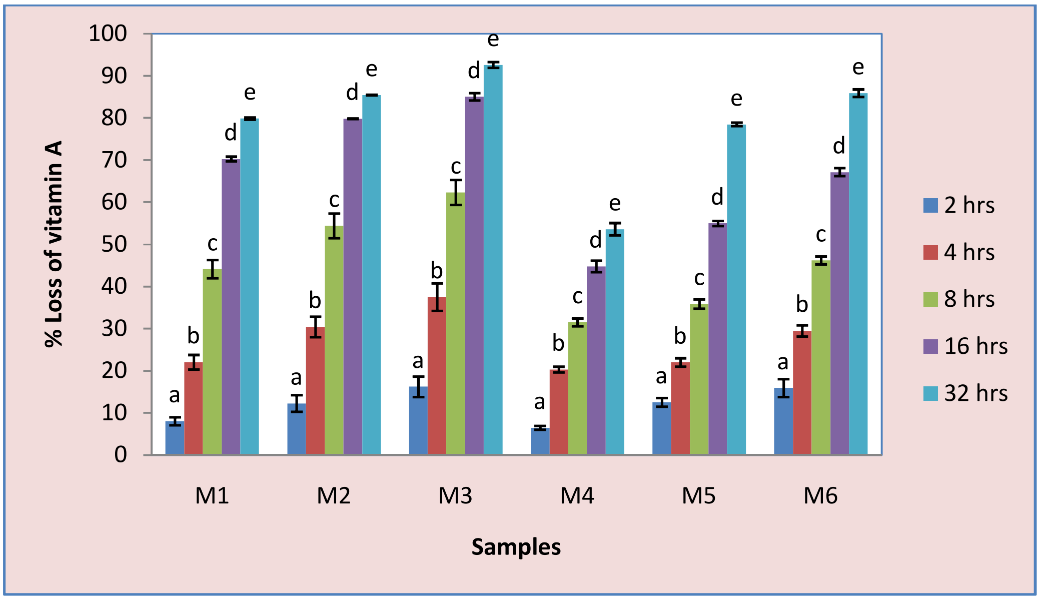

Figure 4.15: Effect of light (4455 lux) on vitamin A retention in milk (% loss)

$^{a-b}$Samples represented with different letters are significantly different (P<0.05) from each other.

Error bars show the variations of three determinations in terms of standard error of mean.

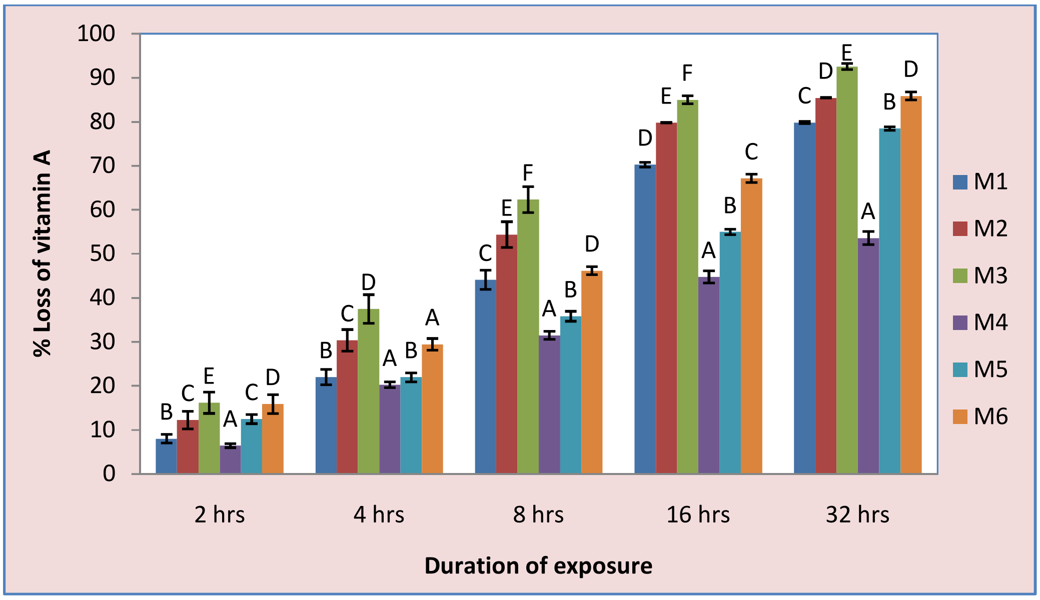

Figure 4.16: Effect of fortification on vitamin A retention in milk exposed to light (4455 lux) (% loss)

$^{A-B}$Samples represented with different letters are significantly different (P<0.05) from each other.

Error bars show the variations of three determinations in terms of standard error of mean.

Loss of vitamin A was higher by exposure of milk to fluorescent light (1485, 2970 and 4455 lux; upto 32 hrs) for iron fortified milk compared to milk without any added iron. This suggests that iron might have played role in accelerating the degradation of vitamin A. Wang and King (1973) reported that iron is known to catalyse the oxidation of milk lipids resulting in the development of an unpleasant odour and flavour (oxidised flavour) and may also cause loss of nutrients such as vitamin E, A and carotene. Milk undergoing oxidation also suffers from loss of the abovesaid nutrients.

In case of FG hydrate fortified milk, the losses were more as compared to FPP soluble fortified milk. This may be due to the fact that FPP soluble is a chelated salt of iron and may interact lesser with the matrix material compared to FG hydrate which is a highly soluble salt of iron and can interact and affect the milk components more than FPP soluble as discussed in section 4.3.1.1.

The intensity of incident light plays a significant role in determining the rate of vitamin loss in milk. It can be concluded from the results that increase in the intensity of light (from 1485 lux to 4455 lux) and with increase in duration of exposure of vitamins to light, losses of vitamin A also increased. Our results were in accordance with Singh *et al.* (1975) and Gaylord *et al.* (1986). Singh *et al.* (1975) reported that when milk was exposed to light at 4ºC in glass containers, the first order rate constant for exposure to an intensity of 4842 lux (450 ft-c) was three times higher than the first order rate constant for milk exposed to 1614 lux (150 ft-c) i.e. degradation of vitamin A was found to be directly proportional to increase in intensity of incident light. Gaylord *et al*. (1986) showed that 53% of the original retinyl palmitate in 2% fat milk after 48 hrs exposure to 1614 lux was retained and rate of retinyl palmitate loss increased with increase in light intensity.

Bector and Rani (1998) reported that the loss of vitamin A in fortified toned milk during exposure to sunlight for a period of 15 min was 15.2% and it increased as period of exposure increased. The losses of vitamin A for exposure periods of 30, 60, 120 and 240 minutes were 23.6, 29.5, 40.8 and 56.0%, respectively. Not much loss of vitamin A content of fortified toned milk was observed when it was exposed to fluorescence light for a period of 15 minutes. However, there were losses in vitamin

A concentration of milk after exposure for more than 15 minutes and these increased as the period of exposure increased. The losses were 6.8, 10.2, 13.2 and 25% for the exposure period of 30, 60, 120 and 240 minutes, respectively.

Thompson and Erdody (1974) reported that vitamin A added to milk is more susceptible to light induced degradation than the natural form present in whole milk. Added vitamin A is known to be destroyed more rapidly by light than naturally. Senyk and Shipe (1981) reported that light sensitivity has to be seen in the reduced fat content of vitamin fortified milk so that light can penetrate deeper than in case of whole milk. Type and composition of carrier fat used in the vitamin fortification mixture and the presence of antioxidant and emulsifiers can also affect vitamin A photostability (Berge *et al*. 1987; Bartholomew and Ogden 1990; Zahar *et al.*1986, 1987, 1992). Bartholomew and Ogden (1990) **s**tudied the comparative stability of naturally occurring vitamin A in milk and that of added externally. They found that vitamin A added to milk is more light sensitive and degraded more rapidly than indigenous vitamin A when concentrated oil solutions of vitamin A and emulsifier were used for fortification.

However, it is to be pointed out that vitamin A used in the present study was in microencapsulated form, and greater stability of added vitamin A compared to inherent vitamin A could be ascribed to the protection of encapsulated vitamin A which might have been provided by the surrounding wall material. This wall material shielded the core material (vitamin A acetate) from the effect of light and other processing conditions.

The mechanism for vitamin A destruction by light is not well understood. Wishner (1964) stated that loss of vitamin A activity results form an opening of the β-ionone ring portion of vitamin A molecule. α-tocopherol, a free radical scavenger, added to the fortifying mixture had no stabilising effect on vitamin A (Berge *et al.* 1987). α-tocopherol's ineffectiveness in stabilising vitamin A indicates that vitamin A loss is not due to autoxidation or even a free radical mechanism. The most probable mechanism is light-induced isomerisation of the vitamin A molecule (Gaylord *et al.* 1985).

Although there was a noticeable loss of vitamin A upon exposure to light, however, minimising light exposure could provide substantial protection to the light-sensitive vitamins in milk.

4.3.1.3 Effect of packaging material on retention of vitamin A in milk

4.3.1.3.1 Effect of storage in polyethylene pouches on retention of vitamin A in milk

All the milk samples were packed in polyethylene pouches and stored under refrigerated conditions (4-7ºC). Vitamin A content decreased significantly ($P<0.05$) with duration of storage in all types of milk samples. Vitamin A content at 0 day was 587.56, 587.56, 591.00, 3053.42, 3053.42 and 3062.73 IU/L for M_1, M_2, M_3, M_4, M_5 and M_6, respectively; after 3 days storage was 536.89, 505.72, 489.92, 3040.58, 3016.06 and 2919.48 IU/L for M_1, M_2, M_3, M_4, M_5 and M_6, respectively, after 7 days was 338.59, 132.12, 108.73, 2473.65, 1852.70 and 1714.90 IU/L for M_1, M_2, M_3, M_4, M_5 and M_6, respectively (table 4.39). M_1, M_4 and M_5 did not show significant decrease in vitamin A content upto 3 days, however, all other samples underwent significant change in vitamin A content even on 3rd day of storage.

There was significant difference ($P<0.05$) in % loss of vitamin A for all milk samples at 3rd, 5th and 7th day of storage (% loss after 3 days was 8.64, 13.94, 17.07, 0.42, 1.22 and 4.67 for M_1, M_2, M_3, M_4, M_5 and M_6, respectively; after 5 days was 20.75, 47.42, 50.90, 11.94, 24.09 and 27.75 for M_1, M_2, M_3, M_4, M_5 and M_6, respectively; and after 7 days was 42.42, 77.60, 81.61, 18.99, 39.33 and 44.02 for M_1, M_2, M_3, M_4, M_5 and M_6, respectively) as shown in figure 4.17.

All the milk samples except M_4 and M_5 differed significantly ($P<0.05$) from each other in terms of vitamin A loss on 3rd day of storage. However, on 5th and 7th day, there was significant difference ($P<0.05$) in vitamin A losses among all milk samples (figure 4.18). The results indicated that there was greater loss of vitamin A in case of FPP soluble fortified milk and FG hydrate fortified milk under same storage conditions and time of storage compared with that occurring in control milk. Thus, indicating that iron played a significant role in accelerating the degradation of vitamin A. In vitamin A fortified milk (M_4), % loss after 7 days storage (18.99%) was much less compared to that occurring in control milk (M_1) (42.42%) as shown in figure 4.18, indicating that added vitamin A (encapsulated) was more stable than the inherent vitamin A present in milk. In case of milk fortified with both vitamin A and iron salt (M_5 and M_6), the losses were more than vitamin A fortified milk. However, the losses were lesser compared to milk fortified only with iron (M_2 and M_3). This strengthened our belief that added (microencapsulated) vitamin A was more stable than vitamin A native to milk.

Table 4.39: Effect of storage in polyethylene pouches at 4-7ºC on vitamin A retention in milk

Storage time→ Sample↓	Vitamin A content in milk (IU/L)			
	0 day	**3rd day**	**5th day**	**7th day**
CTRL (M_1)	587.56 $\pm12.55^{a}$	536.89 $\pm22.97^{a}$	466.00 $\pm33.58^{b}$	338.59 $\pm30.69^{c}$
CTRL+FPP25 (M_2)	587.56 $\pm12.55^{a}$	505.72 $\pm12.36^{b}$	309.18 $\pm12.64^{c}$	132.12 $\pm16.04^{d}$
CTRL+FG25 (M_3)	591.0 $\pm11.17^{a}$	489.92 $\pm4.78^{b}$	290.15 $\pm9.32^{c}$	108.73 $\pm5.72^{d}$
CTRL+VA2500 (M_4)	3053.42 $\pm23.64^{a}$	3040.58 $\pm22.04^{a}$	2688.72 $\pm20.44^{b}$	2473.65 $\pm24.02^{c}$
CTRL+VA2500 +FPP25 (M_5)	3053.42 $\pm23.64^{a}$	3016.06 $\pm25.01^{a}$	2317.91 $\pm17.48^{b}$	1852.70 $\pm28.27^{c}$
CTRL+VA2500 +FG25 (M_6)	3062.73 $\pm28.29^{a}$	2919.48 $\pm39.67^{b}$	2213.05 $\pm57.58^{c}$	1714.90 $\pm43.32^{d}$

Data are presented as means±SEM (n=3).

$^{a-b}$Means with the same superscript in a row are not significantly different (P<0.05) from each other.

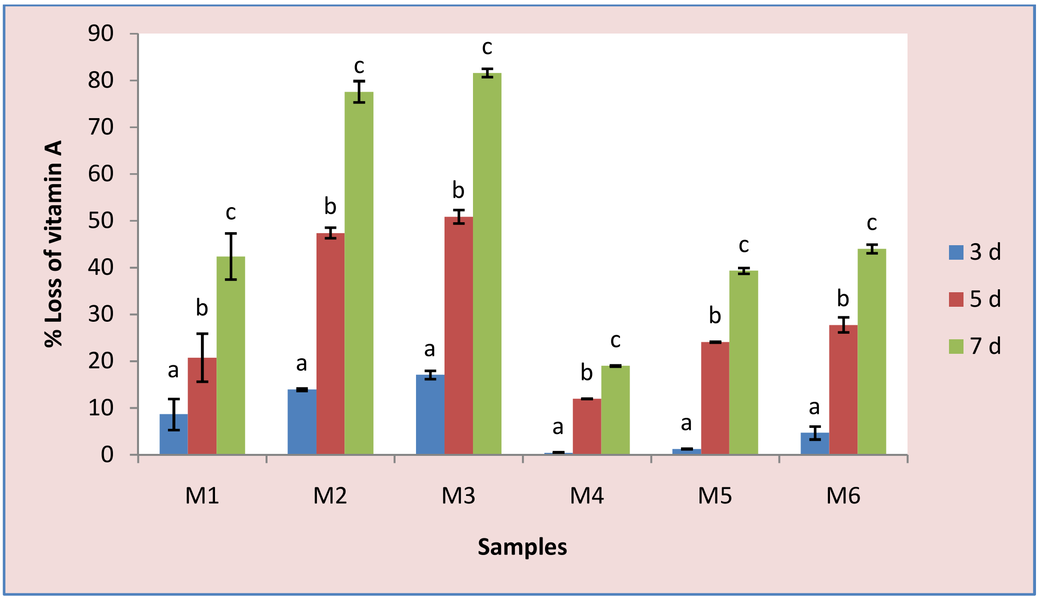

Figure 4.17: Effect of storage in polyethylene pouches on vitamin A retention in milk (% loss)

$^{a\text{-}b}$Samples represented with different letters are significantly different ($P<0.05$) from each other.

Error bars show the variations of three determinations in terms of standard error of mean.

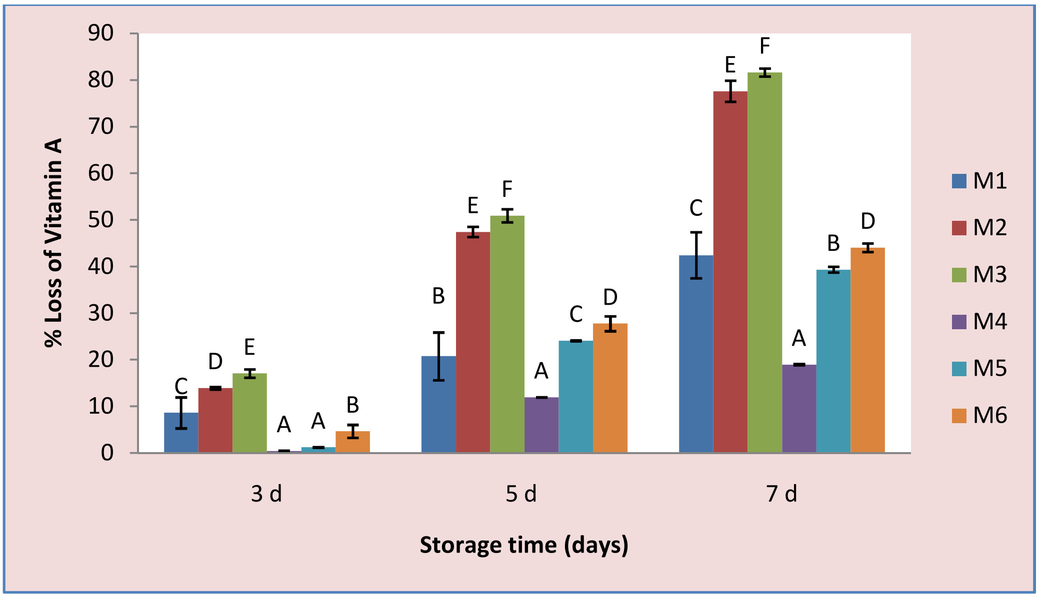

Figure 4.18: Effect of fortification on vitamin A retention in milk stored in polyethylene pouches at 4-7ºC (% loss)

$^{A\text{-}B}$Samples represented with different letters are significantly different ($P<0.05$) from each other.

Error bars show the variations of three determinations in terms of standard error of mean.

4.3.1.3.2 Effect of storage in glass bottles on retention of vitamin A in milk

All the milk samples were packed in glass bottles and stored under refrigerated conditions (4-7ºC). It is evident from table 4.40 that vitamin A content decreased with duration of storage in all types of milk samples. Vitamin A content at 0 day was 604.04, 604.10, 604.22, 3088.51, 3072.81 and 3071.076 IU/L for M_1, M_2, M_3, M_4, M_5 and M_6, respectively; after 3 days storage was 590.42, 546.48, 534.54, 3078.46, 3055.90 and 2966.45± IU/L for M_1, M_2, M_3, M_4, M_5 and M_6, respectively, after 7 days was 468.00, 292.18, 258.32, 2732.87, 2309.83 and 1994.07 IU/L for M_1, M_2, M_3, M_4, M_5 and M_6, respectively. M_1, M_4 and M_5 did not show significant decrease ($P<0.05$) in vitamin A content upto 3 days, however, all other samples underwent significant change in vitamin A content even on 3rd day of storage.

There was significant difference in % loss of vitamin A for all milk samples at 3rd, 5th and 7th day of storage (% loss after 3 days was 2.28, 9.58, 11.57, 0.33, 0.55 and 3.40 for M_1, M_2, M_3, M_4, M_5 and M_6, respectively; after 5 days was 9.17, 32.33, 36.42, 6.34, 13.42 and 19.40 for M_1, M_2, M_3, M_4, M_5 and M_6, respectively; and after 7 days was 22.53, 51.61, 57.23, 11.51, 24.82 and 35.06 for M_1, M_2, M_3, M_4, M_5 and M_6, respectively) as shown in figure 4.19.

All the milk samples except M_4 and M_5 differed significantly from each other in terms of vitamin A loss on 3rd day of storage, while on 5th and 7th day, there was significant difference in vitamin A losses among all milk samples (figure 4.20). The results indicate that there was greater loss of vitamin A in case of FPP soluble fortified milk and FG hydrate fortified milk under same storage conditions and time of storage compared with that occurring in control milk as discussed earlier in section 4.3.1.1 and 4.3.1.2. Iron played a significant role in accelerating the degradation of vitamin A. In vitamin A fortified milk (M_4), % losses after 7 days storage (11.51%) were much less compared to those occurring in control milk (M_1) (22.53%) as shown in figure 4.20, indicating that added vitamin A (encapsulated) was more stable than the inherent vitamin A present in milk. In case of milk fortified with both vitamin A and iron salt, the losses were more than vitamin A fortified milk. However, the losses were lesser compared to milk fortified only with iron. Hence, it is evidently clear that added (microencapsulated) vitamin A was more stable than vitamin A native to milk.

Table 4.40: Effect of storage in glass bottles at 4-7ºC on vitamin A retention in milk

Storage time→ Sample↓	**Vitamin A content in milk (IU/L)**			
	0 day	**3rd day**	**5th day**	**7th day**
CTRL (M$_1$)	604.04 ± 14.63^{a}	590.42 ± 17.62^{a}	548.69 ± 13.75^{b}	468.00 ± 11.99^{c}
CTRL+FPP25 (M$_2$)	604.10 ± 15.45^{a}	546.48 ± 19.74^{b}	408.68 ± 8.69^{c}	292.18 ± 4.29^{d}
CTRL+FG25 (M$_3$)	604.22 ± 16.50^{a}	534.54 ± 19.10^{b}	384.10 ± 9.54^{c}	258.32 ± 4.97^{d}
CTRL+VA2500 (M$_4$)	3088.51 ± 40.82^{a}	3078.46 ± 46.21^{a}	2891.94 ± 11.09^{b}	2732.87 ± 26.31^{c}
CTRL+VA2500 +FPP25 (M$_5$)	3072.81 ± 32.38^{a}	3055.90 ± 29.56^{a}	2660.52 ± 30.60^{b}	2309.83 ± 10.73^{c}
CTRL+VA2500 +FG25 (M$_6$)	3071.07 ± 35.06^{a}	2966.45 ± 27.17^{b}	2475.36 ± 30.33^{c}	1994.07 ± 10.47^{d}

Data are presented as means±SEM (n=3).
[a-b]Means with the same superscript in a row are not significantly different (P<0.05) from each other.

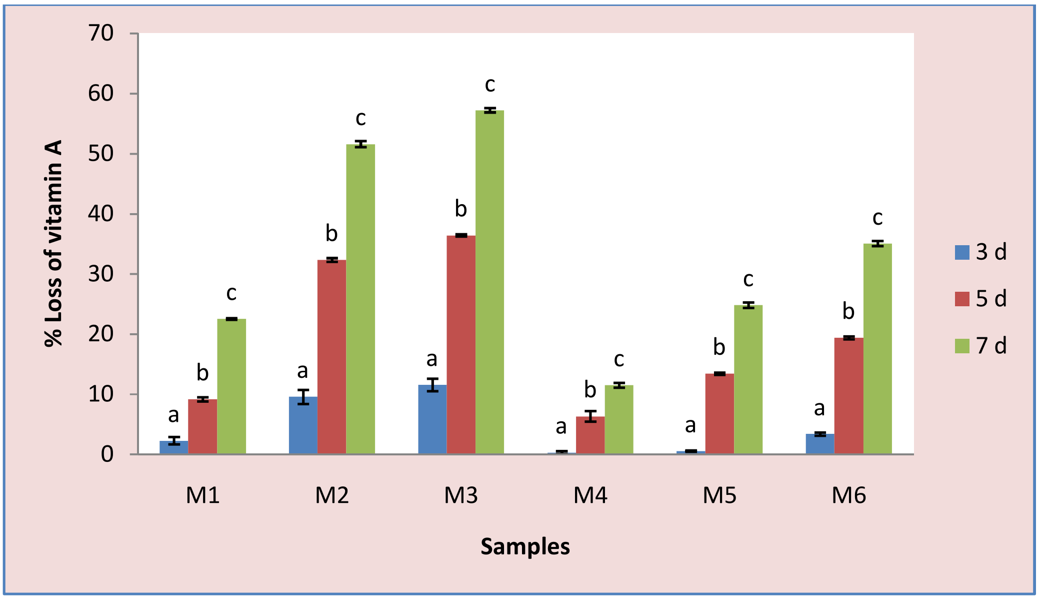

Figure 4.19: Effect of storage in glass bottles on vitamin A retention in milk (% loss)

[a-b]Samples represented with different letters are significantly different (P<0.05) from each other.
Error bars show the variations of three determinations in terms of standard error of mean.

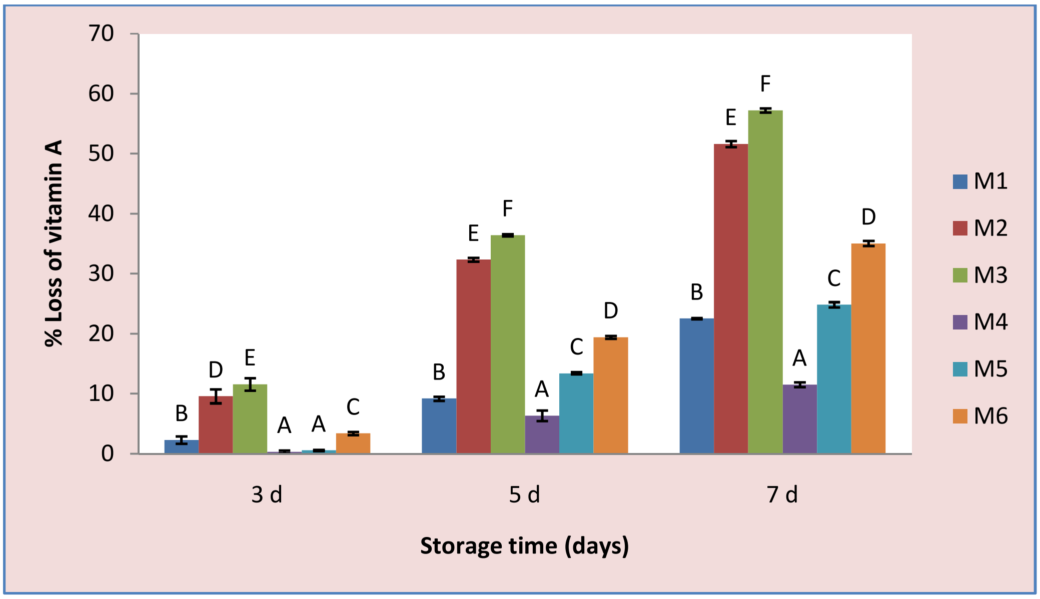

Figure 4.20: Effect of fortification on vitamin A retention in milk stored in glass bottles at 4-7ºC (% loss)

$^{A-B}$Samples represented with different letters are significantly different ($P<0.05$) from each other.

Error bars show the variations of three determinations in terms of standard error of mean.

Loss of vitamin A was higher during storage in both polyethylene pouches and glass bottles for iron fortified milk compared to milk without any added iron. This suggests that iron might have played a role in accelerating the degradation of vitamin A. Wang and King (1973) reported that iron is known to catalyse the oxidation of milk lipids resulting in the development of an unpleasant odour and flavour (oxidised flavour) and may also cause loss of nutrients such as vitamin E, vitamin A and carotene. Milk undergoing oxidation also suffers from loss of the abovesaid nutrients.

In case of FG hydrate fortified milk, the losses were more as compared to FPP soluble fortified milk. This may be due to the fact that FPP soluble is a chelated salt of iron and may interact lesser with the matrix material compared to FG hydrate which is a highly soluble salt of iron and can interact and affect the milk components more than FPP soluble as discussed in earlier sections 4.3.1.1 and 4.3.1.2.

The amount of oxygen in headspace alongwith that dissolved in the milk, would have a deleterious effect on vitamin stability (Melo and Hansen 1978). Vitamin A used for the fortification can be lost by isomerisation and oxidative degradation during storage leading to undesirable changes in flavour and nutritive value of the cereals (Kim *et al*. 2000). The oxidative degradation of vitamin A palmitate may be due to its antioxidant activity. It can quench free radicals and singlet oxygen, which in turn leads to its degradation. If the formation of free radicals (or singlet oxygen) is retarded or removed by other compounds, the degradation of vitamin A palmitate can be reduced. Small amounts of unsaturated fat have been shown to substantially shorten the induction period of vitamin A degradation (Budowski and Bondi 1960).

Our results were in accordance with Bector and Rani (1998) who reported that there was no substantial loss in vitamin A content of fortified toned milk at refrigerated temperature for a storage period of 48 hrs. However, beyond this period, there was loss in vitamin A content and it increased as the period of storage increased. A loss of 3.5% of vitamin A content of fortified toned milk was observed after a storage period of 72 hrs which increased to 10.2% at the end of 96 hrs. Ayyadurai *et al*. (1999) reported that when levels of vitamin A added were 100, 200, 300, 400 and 500 IU per 100 ml, the loss of vitamin A after 7 days were 2.70, 2.60, 2.66, 2.62 and 2.76%, respectively.

However, Maguer and Jackson (1983) reported that vitamin A remained relatively stable up until and beyond the coded pull dates of the pasteurised (12 days) and ultra-high temperature processed (3 months) milk samples stored at 4 and 20ºC, respectively. Examination of pasteurised milk products for vitamin A on the day the products were processed and after 12 days of storage of the same (opened) cartons at 4ºC revealed negligible degradation of 0.6 to 7.2%. Loss of vitamin A in polyethylene packages was higher than that occurring in milks stored in glass bottles (% loss after 7 days in milk stored in polyethylene pouches was 42.42, 77.60, 81.61, 18.99, 39.33 and 44.02 for M_1, M_2, M_3, M_4, M_5 and M_6, respectively; and those stored in glass bottles was 22.53, 51.61, 57.23, 11.51, 24.82 and 35.06 for M_1, M_2, M_3, M_4, M_5 and M_6, respectively).

Our results were contrary to Sauvant *et al*. (2011) who reported that non significant loss of all-trans retinol occurred in milk contained in paperboard boxes, while the loss was significantly lower in plastic containers than in glass. They reported that under low oxygen tension, retinol is considered to be heat resistant. A comparison among glass, plastic and paperboard containers showed non significant loss of all-trans retinol in milk contained in paperboard boxes, while the loss was significantly lower in plastic containers than in glass. In case of present investigation, the difference in stability of vitamin A stored in polyethylene and glass materials could be ascribed to the sorption of vitamin A by polyethylene. Our results can be correlated with the work of Paredes (1996) who indicated that the maximum sorption of vitamin A was 63% by low density polyethylene (LDPE) plastic bottle and up to 55% for by high density polyethylene (HDPE) plastic after one hour of contact with fortified skim milk.

4.3.2 Effect of processing conditions on stability of iron

Minerals are more resistant to manufacturing processes than vitamins. However, they do undergo changes when exposed to heat, air or light. Minerals such as copper, iron and zinc are also affected by moisture and may react with other food components such as proteins and carbohydrates (SUSTAIN 2001). Toned milk was fortified with iron and iron+vitamin A and subjected to different heat treatments viz. pasteurisation (63ºC/15 minutes), boiling and sterilisation (121ºC/15 minutes/15 psi); light exposure treatments (1485, 2970 and 4455 lux/0, 2, 4, 8, 16 and 32 hrs) and storage under refrigerated conditions (4-7ºC) in polyethylene pouches and glass bottles. Effect of these processing conditions on iron retention in control milk (F_1), FPP soluble@25 ppm fortified milk (F_2), FG hydrate@25 ppm fortified milk (F_3), vitamin A acetate@2500 IU/L+FPP soluble@25 ppm fortified milk (F_4) and vitamin A acetate@2500 IU/L+FG hydrate@25 ppm fortified milk (F_5) was then evaluated.

4.3.2.1 Effect of heat treatment on iron retention in milk

There was non significant effect ($P<0.05$) of heat treatments on iron retention in milk in all types of samples. Iron content (in ppm) of raw milk was 0.968±0.05, 25.762, 25.790, 25.760 and 25.760 for F_1, F_2, F_3, F_4 and F_5, respectively; and that of sterilised milk was 0.972, 25.766, 25.818, 25.769 and 25.811 for F_1, F_2, F_3, F_4 and F_5, respectively as shown in table 4.41. Raw, pasteurised, boiled and sterilised milk samples were showing non significant difference in iron content suggesting that iron content of milk samples was not affected by any of the heat treatments given to milk.

This could be due to the fact that minerals are less sensitive than vitamins to physical and chemical factors. Nevertheless, they are reactive and may have potential adverse effects on the sensory properties of milk, and the bioavailability of the mineral form.

Table 4.41: Effect of heat treatment on iron retention in milk

Treatment→ Sample↓	**Iron content in milk (ppm)**			
	Raw milk	**Pasteurised milk**	**Boiled milk**	**Sterilised milk**
CTRL (F_1)	0.968 ± 0.05^a	0.968 ± 0.05^a	0.972 ± 0.05^a	0.972 ± 0.05^a
CTRL+FPP25 (F_2)	25.762 ± 0.14^a	25.761 ± 0.14^a	25.766 ± 0.14^a	25.766 ± 0.14^a
CTRL+FG25 (F_3)	25.790 ± 0.13^a	25.789 ± 0.13^a	25.818 ± 0.14^a	25.818 ± 0.14^a
CTRL+VA2500 +FPP25 (F_4)	25.760 ± 0.14^a	25.760 ± 0.14^a	25.768 ± 0.14^a	25.769 ± 0.14^a
CTRL+VA2500 +FG25 (F_5)	25.789 ± 0.13^a	25.789 ± 0.13^a	25.810 ± 0.13^a	25.811 ± 0.13^a

Data are presented as means±SEM (n=3).
$^{a-b}$Means with the same superscript in a row are not significantly different (P<0.05) from each other.

4.3.2.2 Effect of light intensity (1485, 2970 and 4455 lux) and duration of exposure (0, 2, 4, 8, 16 and 32 hrs) on iron retention in milk

It is evident from the table 4.42, 4.43 and 4.44 that there was non significant effect (P<0.05) of exposure to light (1485, 2970 and 4455 lux) upto 32 hrs duration on iron content of all types of milk samples, suggesting that iron content of milk samples was not affected by light exposure given to milk. The results revealed that exposing milk to very adverse light conditions had no impact on retention of iron content in milk, however, it could act as a catalytic factor for increasing reactivity of iron towards sensitive constituents of milk, especially vitamin A, component of prime interest in present investigation.

Table 4.42: Effect of light (1485 lux) on iron retention in milk

Duration of exposure→ Sample↓	Iron content in milk (ppm)					
	0 hr	2 hrs	4 hrs	8 hrs	16 hrs	32 hrs
CTRL (F_1)	0.876 ±0.03^{a}	0.876 ±0.03^{a}	0.875 ±0.03^{a}	0.876 ±0.03^{a}	0.875 ±0.029^{a}	0.875 ±0.029^{a}
CTRL+FPP25 (F_2)	25.763 ±0.07^{a}	25.763 ±0.07^{a}	25.762 ±0.07^{a}	25.763 ±0.07^{a}	25.763 ±0.071^{a}	25.763 ±0.07^{a}
CTRL+FG25 (F_3)	25.582 ±0.13^{a}	25.580 ±0.12^{a}	25.581 ±0.12^{a}	25.577 ±0.13^{a}	25.577 ±0.13^{a}	25.577 ±0.13^{a}
CTRL+VA2500 +FPP25 (F_4)	25.763 ±0.07^{a}	25.761 ±0.07^{a}	25.757 ±0.07^{a}	25.756 ±0.07^{a}	25.757 ±0.07^{a}	25.7616 ±0.07^{a}
CTRL+VA2500 +FG25 (F_5)	25.576 ±0.12^{a}	25.572 ±0.12^{a}	25.573 ±0.12^{a}	25.573 ±0.12^{a}	25.572 ±0.12^{a}	25.571 ±0.12^{a}

Data are presented as means±SEM (n=3).
$^{a\text{-}b}$Means with the same superscript in a row are not significantly different ($P<0.05$) from each other.

Table 4.43: Effect of light (2970 lux) on iron retention in milk

Duration of exposure→ Sample↓	Iron content in milk (ppm)					
	0 hr	2 hrs	4 hrs	8 hrs	16 hrs	32 hrs
CTRL (F_1)	0.782 ±0.08^{a}	0.779 ±0.08^{a}	0.780 ±0.08^{a}	0.7780 ±0.08^{a}	0.779 ±0.08^{a}	0.780 ±0.08^{a}
CTRL+FPP25 (F_2)	25.717 ±0.09^{a}	25.701 ±0.09^{a}	25.709 ±0.09^{a}	25.709 ±0.09^{a}	25.713 ±0.09^{a}	25.710 ±0.09^{a}
CTRL+FG25 (F_3)	25.613 ±0.04^{a}	25.911 ±0.04^{a}	25.607 ±0.04^{a}	25.607 ±0.04^{a}	25.607 ±0.04^{a}	25.598 ±0.05^{a}
CTRL+VA2500 +FPP25 (F_4)	25.726 ±0.09^{a}	25.722 ±0.09^{a}	25.724 ±0.09^{a}	25.722 ±0.09^{a}	25.717 ±0.09^{a}	25.724 ±0.09^{a}
CTRL+VA2500 +FG25 (F_5)	25.612 ±0.04^{a}	25.610 ±0.04^{a}	25.605 ±0.04^{a}	25.607 ±0.04^{a}	25.610 ±0.04^{a}	25.610 ±0.04^{a}

Data are presented as means±SEM (n=3).
$^{a\text{-}b}$Means with the same superscript in a row are not significantly different ($P<0.05$) from each other.

Table 4.44: Effect of light (4455 lux) on iron retention in milk

Duration of exposure→ Sample↓	**Iron content in milk (ppm)**					
	0 hrs	**2 hrs**	**4 hrs**	**8 hrs**	**16 hrs**	**32 hrs**
CTRL (F_1)	0.897 $\pm0.02^a$	0.895 $\pm0.02^a$	0.892 $\pm0.02^a$	0.891 $\pm0.02^a$	0.889 $\pm0.02^a$	0.891 $\pm0.02^a$
CTRL+FPP25 (F_2)	25.835 $\pm0.04^a$	25.831 $\pm0.04^a$	25.825 $\pm0.04^a$	25.831 $\pm0.05^a$	25.828 $\pm0.05^a$	25.829 $\pm0.05^a$
CTRL+FG25 (F_3)	25.657 $\pm0.09^a$	25.657 $\pm0.09^a$	25.654 $\pm0.09^a$	25.656 $\pm0.09^a$	25.656 $\pm0.09^a$	25.653 $\pm0.09^a$
CTRL+VA2500 +FPP25 (F_4)	25.812 $\pm0.04^a$	25.805 $\pm0.04^a$	25.806 $\pm0.04^a$	25.807 $\pm0.04^a$	25.806 $\pm0.04^a$	25.804 $\pm0.04^a$
CTRL+VA2500 +FG25 (F_5)	25.668 $\pm0.09^a$	25.661 $\pm0.09^a$	25.664 $\pm0.09^a$	25.660 $\pm0.09^a$	25.664 $\pm0.09^a$	25.665 $\pm0.09^a$

Data are presented as means±SEM (n=3).
$^{a-b}$Means with the same superscript in a row are not significantly different ($P<0.05$) from each other.

4.3.2.3 Effect of packaging material on retention of iron in milk

4.3.2.3.1 Effect of storage in polyethylene pouches on retention of iron

It is clear from the table 4.45 that there was non significant effect ($P<0.05$) of storage in polyethylene pouches under refrigerated conditions (4-7^{o}C) on iron content of all types of milk samples. Iron content (in ppm) on 0 day of storage was 0.849, 25.687, 25.601, 25.685 and 25.685 for F_1, F_2, F_3, F_4 and F_5, respectively; and on 7th day was 0.816, 25.672, 25.622, 25.644 and 25.601 for F_1, F_2, F_3, F_4 and F_5, respectively.

Table 4.45: Effect of storage in polyethylene pouches on iron retention in milk

Storage time→ Sample↓	Iron Content in milk (ppm)			
	0 day	3rd day	5th day	7th day
CTRL (F_1)	0.849 ±0.05^a	0.816 ±0.05^a	0.815 ±0.05^a	0.816 ±0.05^a
CTRL+FPP25 (F_2)	25.687 ±0.08^a	25.624 ±0.06^a	25.655 ±0.04^a	25.672 ±0.07^a
CTRL+FG25 (F_3)	25.601 ±0.02^a	25.580 ±0.02^a	25.567 ±0.03^a	25.622 ±0.04^a
CTRL+VA2500 +FPP25 (F_4)	25.685 ±0.08^a	25.651 ±0.09^a	25.616 ±0.08^a	25.644 ±0.07^a
CTRL+VA2500 +FG25 (F_5)	25.600 ±0.02^a	25.585 ±0.04^a	25.551 ±0.03^a	25.601 ±0.02^a

Data are presented as means±SEM (n=3).
$^{a-b}$Means with the same superscript in a row are not significantly different ($P<0.05$) from each other.

4.3.2.3.2 Effect of storage in glass bottles on retention of iron

There was non significant effect ($P<0.05$) of storage in glass bottles under refrigeration conditions on iron content of all types of milk samples. Iron content (in ppm) on 0 day of storage was 0.915±0.016, 25.652±0.11, 25.652±0.07, 25.654±0.11 and 25.645±0.07 for F_1, F_2, F_3, F_4 and F_5, respectively; and on 7th day was 0.911±0.02, 25.631±0.11, 25.649±0.07, 25.650±0.11 and 25.641±0.07 for F_1, F_2, F_3, F_4 and F_5, respectively as shown in table 4.46.

Table 4.46: Effect of storage in glass bottles on iron retention in milk

Storage time→ Sample↓	**Iron content in milk (ppm)**			
	0 day	**3rd day**	**5th day**	**7th day**
CTRL (F_1)	0.915± 0.01^a	0.911± 0.02^a	0.911± 0.02^a	0.911± 0.02^a
CTRL+FPP25 (F_2)	25.652± 0.11^a	25.634± 0.11^a	25.638± 0.11^a	25.631± 0.11^a
CTRL+FG25 (F_3)	25.652± 0.07^a	25.646± 0.07^a	25.647± 0.07^a	25.649± 0.07^a
CTRL+VA2500 +FPP25 (F_4)	25.654± 0.11^a	25.649± 0.11^a	25.650± 0.11^a	25.650± 0.11^a
CTRL+VA2500 +FG25 (F_5)	25.645± 0.07^a	25.640± 0.07^a	25.641± 0.07^a	25.641± 0.07^a

Data are presented as means±SEM (n=3).
$^{a-b}$Means with the same superscript in a row are not significantly different (P<0.05) from each other.

All the milk samples showed non significant difference (P<0.05) in iron content throughout the duration of storage, suggesting that iron content of milk samples was not affected by storage and the packaging material i.e. glass or polyethylene under the conditions used in present study. Our results were in accordance with Ayyadurai *et al*. (1999) who determined the stability of iron in fortified milk after storage at 5ºC. The loss of iron content in different concentrations of 10, 20 and 30 mg/100 ml were 0.25, 0.23 and 0.20%, respectively which were considered negligible suggesting that non significant loss of iron occurred upon storage.

It can be inferred from the results that iron content of milk did not change significantly during different treatments given to milk i.e. heat treatments (pasteurisation, boiling and sterilisation); exposure to fluorescent light and storage in different packaging materials (polyethylene pouches and glass bottles). Iron in the control and fortified milk was stable towards the usual treatments given to milk and did not undergo any decomposition.

4.4 Storage studies

Milk samples supplemented with iron salts (FPP soluble and FG hydrate@25 ppm) and vitamin A acetate@2500 IU/L alongwith control samples were stored at refrigerated temperature (4-7ºC) for 7 days. All milk samples viz. control milk, VA@2500 IU/L+FPP soluble@25 ppm fortified milk (VA2500+FPP25) and VA@2500 IU/L+FG hydrate@25 ppm fortified milk (VA2500+FG25) were regularly evaluated and results are presented here under.

4.4.1 Change in sensory acceptability of milk during storage

4.4.1.1 Sensory scores of milk on 0 day of storage

The overall sensory scores obtained for control milk, VA2500+FPP25 and VA2500+FG25 were 97.69, 97.55 and 97.45 on 0 day of storage (table 4.47). Statistically, the control and fortified milk samples were not significantly different ($P<0.05$) in the sensory aspects i.e. colour and appearance, odour, taste, mouthfeel and total scores.

Table 4.47: Sensory scores of fortified milk on 0 day of storage

Characteristics	**Maximum score**	**Sample**		
		Control	**VA2500+FPP25**	**VA2500+FG25**
Colour and appearance	10	9.67 ± 0.04^a	9.64 ± 0.04^a	9.63 ± 0.04^a
Odour	20	19.59 ± 0.05^a	19.57 ± 0.05^a	19.54 ± 0.05^a
Taste	40	38.92 ± 0.11^a	38.85 ± 0.11^a	38.81 ± 0.11^a
Mouthfeel	30	29.51 ± 0.05^a	29.49 ± 0.05^a	29.47 ± 0.05^a
Total score	100	97.69 ± 0.17^a	97.55 ± 0.17^a	97.45 ± 0.17^a

Data are presented as means±SEM (n=30).

$^{a-b}$Means with the same superscript in a row are not significantly different ($P<0.05$) from each other.

4.4.1.2 Sensory scores of milk on 3rd day of storage

The overall sensory scores obtained for control milk, VA2500+FPP25 and VA2500+FG25 were 96.09, 95.88 and 95.76 on 3rd day of storage (table 4.48). Statistically, the control and fortified milk samples were not significantly different ($P<0.05$) in all sensory aspects i.e. colour and appearance, odour, taste and mouthfeel. However, in terms of total scores, VA2500+FG25 showed significant difference ($P<0.05$) from control milk, but not from VA2500+FPP25.

Table 4.48: Sensory scores of fortified milk on 3rd day of storage

Characteristics	Maximum score	Sample		
		Control	VA2500+FPP25	VA2500+FG25
Colour and appearance	10	9.66±0.03^{a}	9.63±0.03^{a}	9.6±0.03^{a}
Odour	20	19.04±0.06^{a}	18.97±0.06^{a}	18.95±0.06^{a}
Taste	40	38.53±0.07^{a}	38.49±0.07^{a}	38.46±0.07^{a}
Mouthfeel	30	28.85±0.06^{a}	28.79±0.06^{a}	28.75±0.06^{a}
Total score	100	96.09±0.12^{a}	95.88±0.12^{ab}	95.76±0.12^{b}

Data are presented as means±SEM (n=30).
$^{a-b}$Means with the same superscript in a row are not significantly different (P<0.05) from each other.

4.4.1.3 Sensory scores of milk on 5th day of storage

The overall sensory scores obtained for control milk, VA2500+FPP25 and VA2500+FG25 were 93.55, 93.33 and 93.17 on 5th day of storage (table 4.49). Statistically, control and VA2500+FPP25 showed non significant different (P<0.05) in the sensory aspects i.e. colour and appearance, odour, taste and mouthfeel. VA2500+FG25 differed significantly (P<0.05) from control milk but not from VA2500+FPP25 in terms of taste and total scores.

Table 4.49: Sensory scores of fortified milk on 5th day of storage

Characteristics	Maximum score	Sample		
		Control	VA2500+FPP25	VA2500+FG25
Colour and appearance	10	9.39±0.04^{a}	9.36±0.04^{a}	9.32±0.04^{a}
Odour	20	18.28±0.06^{a}	18.22±0.06^{a}	18.18±0.06^{a}
Taste	40	37.55±0.05^{a}	37.47±0.05^{ab}	37.42±0.06^{b}
Mouthfeel	30	28.34±0.04^{a}	28.29±0.05^{a}	28.25±0.05^{a}
Total score	100	93.55±0.11^{a}	93.33±0.11^{ab}	93.17±0.11^{b}

Data are presented as means±SEM (n=30).
$^{a-b}$Means with the same superscript in a row are not significantly different (P<0.05) from each other.

4.4.1.4 Sensory scores of milk on 7th day of storage

The overall sensory scores obtained for control milk, VA2500+FPP25 and VA2500+FG25 were 92.00, 90.76 and 90.38 on 7th day of storage (table 4.50). Statistically, the control and VA2500+FPP25 were non significantly different (P<0.05) in terms of colour and appearance, odour and mouthfeel. However, there was significant difference (P<0.05) in taste and total scores of control milk and

VA2500+FPP25. VA2500+FG25 showed significant difference (P<0.05) from control milk in all sensory aspects except colour and appearance. The fortified milk samples differed from each other in all sensory attributes except colour and appearance and taste.

Table 4.50: Sensory scores of fortified milk on 7th day of storage

Characteristics	Maximum score	Sample		
		Control	**VA2500+FPP25**	**VA2500+FG25**
Colour and appearance	10	9.23±0.03^{a}	9.19±0.03^{a}	9.18±0.03^{a}
Odour	20	18.05±0.04^{a}	18.01±0.05^{a}	17.86±0.05^{b}
Taste	40	36.58±0.09^{a}	35.49±0.09^{b}	35.37±0.09^{b}
Mouthfeel	30	28.13±0.07^{a}	28.07±0.07^{a}	27.97±0.07^{b}
Total score	100	92.00±0.08^{a}	90.76±0.09^{b}	90.38±0.1^{c}

Data are presented as means±SEM (n=30).
$^{a-b}$Means with the same superscript in a row are not significantly different (P<0.05) from each other.

The results of sensory analysis after 7 days storage revealed that the fortified milk samples had good sensory acceptability till 7 days. The total sensory scores of fortified and control milk samples showed slight but statistically significant difference on 5th and 7th day (for VA2500+FG25), however the values were comparable to control suggesting the good sensory acceptability of fortified milk samples up to 7 days.

Our results were in accordance with Xia and Xu (2005) who fortified fluid whole milk with ferrous sulphate liposomes (@15 ppm iron) and during one week storage at 4ºC, observed that control and fortified milk were very stable without any sign of precipitation and coagulation and fortified milk did not differ greatly from control milk in colour and flavour. Ayyadurai *et al*. (1999) also reported that there were no noticeable changes with regard to flavour and acceptability scores in ferric ammonium citrate and vitamin A palmitate fortified milk after seven days storage at 5ºC. Douglas *et al.* (1981) reported that sodium ferric pyrophosphate, ferripolyphosphate and ferripolyphosphate-whey protein complex produced little or no off-colour change in chocolate milk initially or after 2 weeks storage. All samples containing ferric compounds (sodium ferric pyrophosphate, ferripolyphosphate,

ferripolyphosphate-whey protein complex, ferric citrate, ferric choline citrate and ferric ammonium citrate) as compared to the control produced little or no off-flavour in chocolate milks initially or after 7 and 14 days. Wang and King (1973) reported that ferric ammonium citrate fortified milk (@15 ppm iron) was as stable as control in terms of flavour scores after 7 days storage and there was no statistically significant difference in flavour between the control and ferric ammonium citrate fortified milk. Ferric ammonium sulphate and ferric choline chloride resulted in detectable oxidised flavour after 7 days storage and their flavour scores were significantly different from control. Demott (1971) reported that ferric pyrophosphate and ferric phosphate (added @5.2 and 10.4 ppm iron) caused only minor off-flavours in whole and skim milk after 5 days storage at 4ºC.

4.4.2 Change in pH of milk during storage

pH profile of vitamin A+iron fortified milk and corresponding control milk is presented in table 4.51 and figure 4.21. On 0 day, pH of all the milk samples i.e. control, VA2500+FPP25 and VA2500+FG25 did not show any change. On 3rd day of storage, pH of VA2500+FG25 showed significant difference ($P<0.05$) from control milk, but not with VA2500+FPP25. pH of control milk showed significant change ($P<0.05$) on 7th day of storage (6.81 on 0 day and 6.78 on 7th day). After 5 days storage duration, there was significant difference ($P<0.05$) in pH of VA2500+FPP25 (6.81 on 0 day and 6.78 on 5th day) and VA2500+FG25 (6.80 on 0 day and 6.77 on 5th day). In case of FG hydrate fortified milk, the magnitude of decrease in pH was more compared to FPP soluble fortified milk. This may be due to the fact that FPP soluble is a chelated salt of iron and may interact lesser with the matrix material compared to FG hydrate which is a highly soluble salt of iron and can interact and affect the milk components more than FPP soluble.

The present findings were in agreement with findings of Rosenthal *et al*. (1993) who had reported that there was decrease in pH of milk samples; and pH of fortified milk kept on decreasing throughout storage of 7 days, however magnitude of decrease in pH of ferrous lactate (5 mg/100ml) fortified milk and control milk was comparable throughout the duration of storage. Gaucheron *et al*. (1997a) reported that there was reduction in pH of skim milk after addition of $FeCl_2$ and $FeCl_3$. This might be due to release of micellar bound H^+ ions. Ravikiran *et al*. (1977) had reported earlier that there was no detrimental effect on pH after addition of ferric lactose and ascorbic acid. The present findings also show that there was slight but no detrimental effect of iron and vitamin A addition on pH of milk.

Table 4.51: Effect of storage at 4-7^{o}C on the pH of fortified milk

Storage time→ **Sample↓**	**0 day**	**3rd day**	**5th day**	**7th day**
Control	6.81 ± 0.03^{aA}	6.81 ± 0.02^{aA}	6.80 ± 0.03^{abA}	6.78 ± 0.03^{bA}
VA2500+FPP25	6.81 ± 0.03^{aA}	6.80 ± 0.02^{abAB}	6.78 ± 0.02^{bcAB}	6.76 ± 0.02^{cAB}
VA2500+FG25	6.80 ± 0.03^{aA}	6.78 ± 0.02^{abB}	6.77 ± 0.02^{bcB}	6.75 ± 0.02^{cB}

Data are presented as means±SEM (n=3).
$^{a-b}$Means within rows with different lowercase superscript are significantly different (P<0.05) from each other.
$^{A-B}$Means within columns with different uppercase superscript are significantly different (P<0.05) from each other.

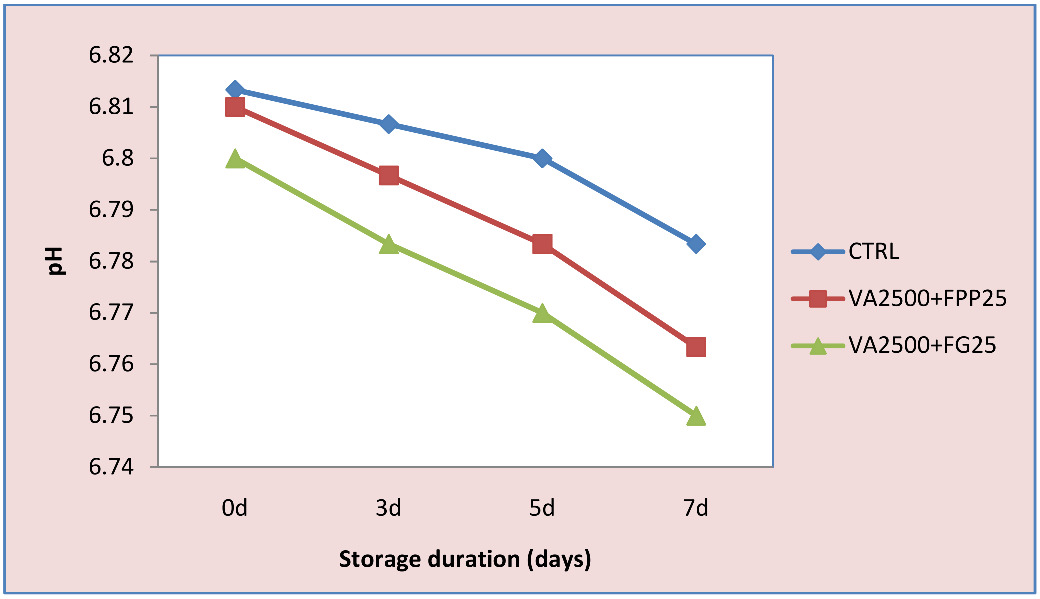

Figure 4.21: Effect of storage at 4-7^{0} C on the pH of fortified milk

4.4.3 Change in titratable acidity of milk

The changes in titratable acidity (% LA) of control as well as vitamin A+iron fortified milk during storage are represented in table 4.52 and figure 4.22. Acidity of control milk showed significant change (P<0.05) on 7th day of storage (0.138% on 0 day and 0.140% on 7th day). After 5 days storage, there was significant difference (P<0.05) in acidity of VA2500+FPP25 (0.140% on 0 day and 0.141% on 5th day) and

VA2500+FG25 (0.140% on 0 day and 0.142% on 5th day). On 0 day, there was slight but significant difference ($P<0.05$) in acidity of control and fortified milk samples, however, the fortified milk samples i.e. VA2500+FPP25 and VA2500+FG25 had similar acidity. From 3rd day of storage onwards, all the samples exhibited significant difference ($P<0.05$) in acidity. The magnitude of difference in acidity of control and fortified milk samples was slightly higher for ferrous gluconate hydrate than ferric pyrophosphate soluble. This may be due to the fact that FPP soluble is a chelated salt of iron and may interact lesser with the matrix material compared to FG hydrate which is a highly soluble salt of iron and can interact and affect the milk components more than FPP soluble. The present findings were in agreement with Ravikiran *et al*. (1977) who had reported that there was not much difference in acidity of control and fortified milk with ferric lactose after 24 hrs of refrigerated storage.

Table 4.52: Effect of storage in 4-7ºC on the acidity (% LA) of fortified milk

Storage time→ **Sample↓**	**0 day**	**3rd day**	**5th day**	**7th day**
Control	0.138 ± 0.001^{aA}	0.138 ± 0.001^{aA}	0.139 ± 0.001^{abA}	0.14 ± 0.001^{bA}
VA2500+FPP25	0.140 ± 0.001^{aB}	0.140 ± 0.001^{aB}	0.141 ± 0.001^{bB}	0.143 ± 0.001^{cB}
VA2500+FG25	0.140 ± 0.001^{aB}	0.141 ± 0.001^{abC}	0.142 ± 0.001^{bC}	0.144 ± 0.001^{cC}

Data are presented as means±SEM (n=3).

$^{a\text{-}b}$Means within rows with different lowercase superscript are significantly different ($P<0.05$) from each other.

$^{A\text{-}B}$Means within columns with different uppercase superscript are significantly different ($P<0.05$) from each other.

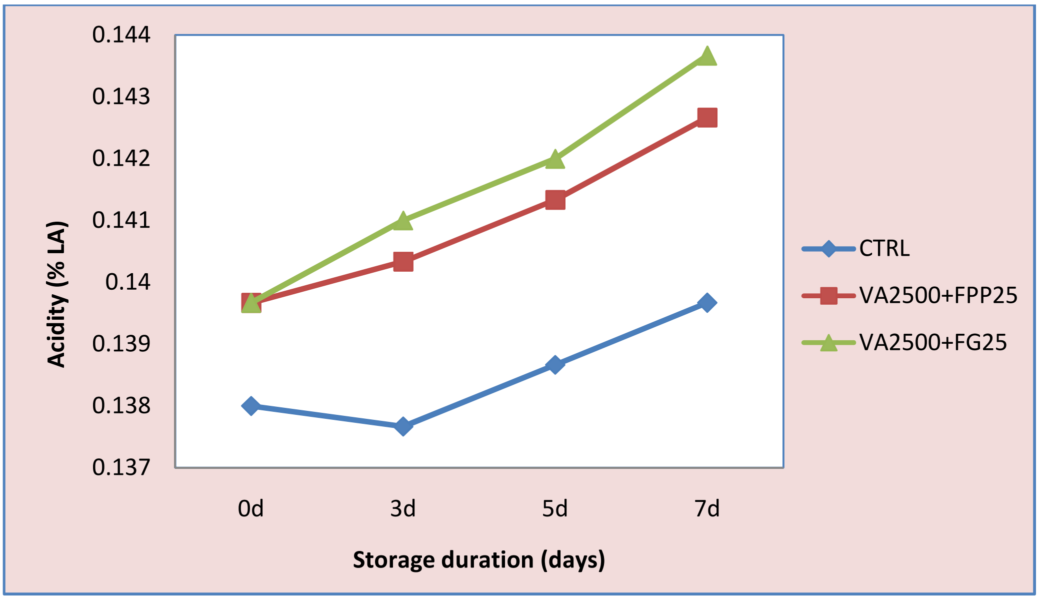

Figure 4.22: Effect of storage at 4-7ºC on the acidity (% LA) of fortified milk

4.4.4 Change in TBA value of milk during storage

The changes in TBA value of control as well as vitamin A+iron fortified milk during storage are represented in table 4.53 and figure 4.23. TBA value of control and fortified milk samples showed significant increase ($P<0.05$) even on 3rd day of storage (0.018 on 0 day and 0.021 on 3rd day for control milk; 0.019 on 0 day and 0.022 on 3rd day for VA2500+FPP25; 0.020 on 0 day and 0.023 on 3rd day for VA2500+FG25). On 0 day, all the samples exhibited significant difference ($P<0.05$) in TBA value (0.018, 0.019 and 0.020 for control, VA2500+FPP25 and VA2500+FG25, respectively).

Our results were in accordance with Wang and King (1973) who reported that there was non significant difference in control and ferric ammonium citrate fortified milk (@10 ppm iron) initially or after 7 days storage, however, ferric ammonium sulphate and ferric choline chloride fortified milk brought significant changes in TBA value of milk even on 0 day of storage. However, Saini *et al*. (1987) observed that there were negligible differences in the TBA values of 48 and 96 hrs stored pasteurised milks fortified with 10 ppm of iron as ferrous and ferric iron with or without ascorbic acid.

Table 4.53: Effect of storage at 4-7ºC on the TBA value of fortified milk

Storage time→ Sample↓	**0 day**	**3rd day**	**5th day**	**7th day**
Control	0.018 ± 0.0003^{aA}	0.021 ± 0.0003^{bA}	0.024 ± 0.0003^{cA}	0.026 ± 0.0003^{dA}
VA2500+FPP25	0.019 ± 0.0003^{aB}	0.022 ± 0.0006^{bB}	0.025 ± 0.0003^{cB}	0.028 ± 0.0006^{dB}
VA2500+FG25	0.020 ± 0.0003^{aC}	0.023 ± 0.0003^{bC}	0.026 ± 0.0006^{cC}	0.033 ± 0.0015^{dC}

Data are presented as means±SEM (n=3).

$^{a\text{-}b}$Means within rows with different lowercase superscript are significantly different ($P<0.05$) from each other.

$^{A\text{-}B}$Means within columns with different uppercase superscript are significantly different ($P<0.05$) from each other.

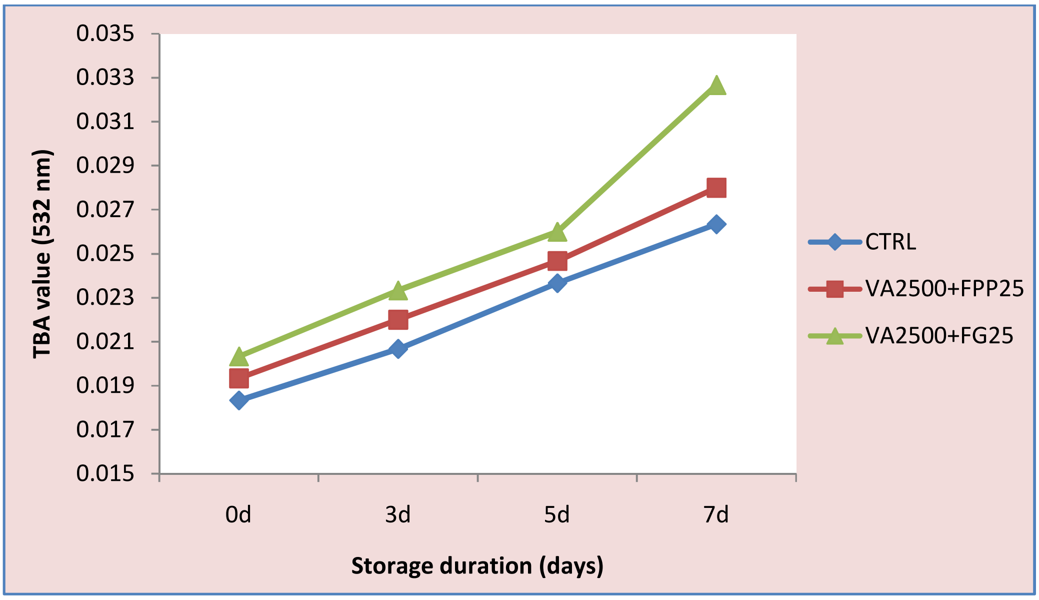

Figure 4.23: Effect of storage at 4-7ºC on the TBA value of fortified milk

4.4.5 Oxidative stability as affected by storage in polyethylene pouches and glass bottles

Since trace elements like iron are known to catalyse the oxidation of lipids, storage stability of ghee prepared from iron fortified milk was studied at refrigerated temperature (4-7ºC). Control milk, vitamin A acetate@2500 IU/L+FPP soluble@25 ppm fortified milk (VA2500+FPP25) and vitamin A acetate@2500 IU/L+FG hydrate@25 ppm fortified milk (VA2500+FG25) were stored in polyethylene pouches and glass bottles at refrigerated temperature (4-7ºC) for 7 days. Samples were drawn at definite time intervals (on 0 day, 3rd day, 5th day and 7th day), cream was separated from milk samples and clarified into ghee by direct creamery method. These ghee samples were analysed for peroxide value, % free fatty acids, TBA value and induction period (oxidative stability index) by Rancimat method.

4.4.5.1 Change in peroxide value during storage

Peroxide value is the measure of hydroperoxides formed during autoxidation. Rao and Ramamurthy (1987) reported that there is simultaneous formation and breakdown of peroxides leading to accumulation of monocarbonyls during lipid oxidation. Peroxide value represents primary reaction products of lipid oxidation, which can be measured by their ability to liberate iodine from potassium iodide. It is considered to represent the quantity of active oxygen (in mg) contained in 1 g of lipid. In our case, samples were drawn on 0 day, 3rd day, 5th day and 7th day of storage.

4.4.5.1.1 Change in peroxide value during storage in polyethylene pouches

It is evident from table 4.54 and figure 4.24 that there was significant difference ($P<0.05$) in peroxide value of ghee samples even on 3rd day of storage (peroxide value in millimoles of oxygen per kg of fat was 0.000 on 0 day and 0.025 on 3rd day for control milk; 0.000 on 0 day and 0.032 on 3rd day for VA2500+FPP25; 0.000 on 0 day and 0.037±0.002 on 3rd day for FG2500+VA2500). On 0 day, there was no difference in the peroxide values of all the ghee samples. On 3rd day, ghee samples from fortified milk differed significantly ($P<0.05$) from control in terms of peroxide value but not from each other (peroxide value was 0.025, 0.032 and 0.037 millimoles of oxygen per kg of fat for control, VA2500+FPP25 and VA2500+FG25). On 5th day, all the ghee samples differed ($P<0.05$) from each other in terms of peroxide value (0.057, 0.075 and 0.087 millimoles of oxygen per kg of fat for control, VA2500+FPP25 and VA2500+FG25).

Table 4.54: Effect of storage in polyethylene pouches at 4-7ºC on peroxide value (millimoles of oxygen per kg fat) of ghee prepared from fortified milk

Storage time→ Sample↓	**0 day**	**3rd day**	**5th day**	**7th day**
Control	0.000 ± 0.000^{aA}	0.025 ± 0.002^{bA}	0.057 ± 0.005^{cA}	0.085 ± 0.003^{dA}
VA2500+FPP25	0.000 ± 0.000^{aA}	0.032 ± 0.003^{bB}	0.075 ± 0.004^{cB}	0.173 ± 0.019^{dB}
VA2500+FG25	0.000 ± 0.000^{aA}	0.037 ± 0.002^{bB}	0.087 ± 0.003^{cC}	0.227 ± 0.005^{dC}

Data are presented as means±SEM (n=3).

$^{a-b}$Means within rows with different lowercase superscript are significantly different ($P<0.05$) from each other.

$^{A-B}$Means within columns with different uppercase superscript are significantly different ($P<0.05$) from each other.

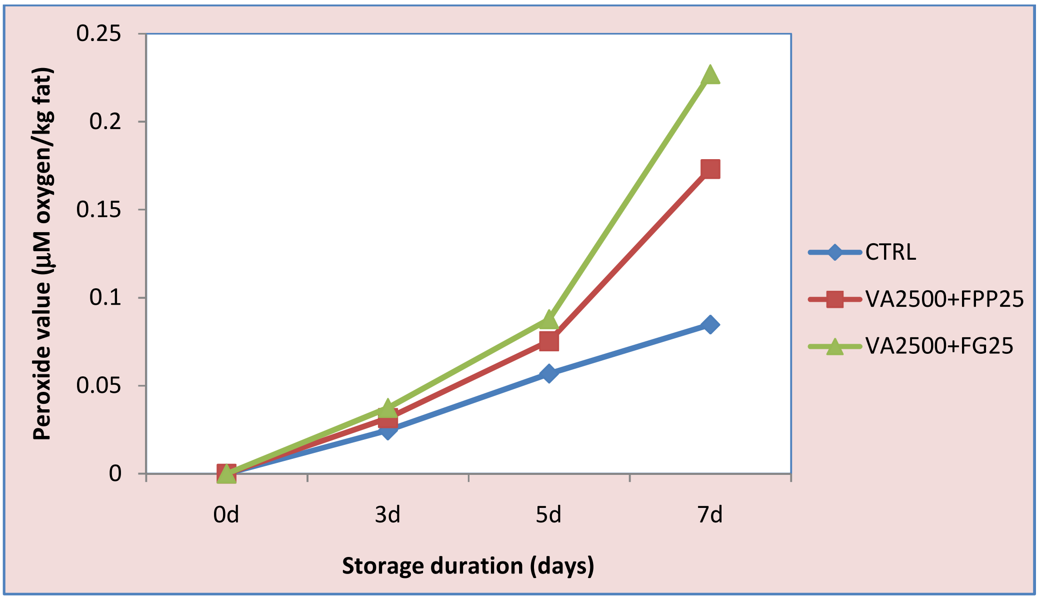

Figure 4.24: Effect of storage in polyethylene pouches at 4-7ºC on peroxide value of ghee prepared from fortified milk

4.4.5.1.2 Change in peroxide value during storage in glass bottles

It is evident from table 4.55 and figure 4.25 that there was significant difference ($P<0.05$) in peroxide value of ghee samples even on 3rd day of storage (0.000 on 0 day and 0.024 on 3rd day for control; 0.000 on 0 day and 0.029 on 3rd day for VA2500+FPP25; 0.000 on 0 day and 0.035 on 3rd day for VA2500+FG25).

On 0 day, there was no difference in the peroxide values of all the samples. On 3rd day, peroxide value of control and VA2500+FPP25 did not show any significant difference ($P<0.05$) (peroxide value in millimoles of O_2 per kg fat was 0.024 for control and 0.029 for VA2500+FPP25), however, VA2500+FG25 differed significantly ($P<0.05$) from control ghee and VA2500+FPP25 in terms of peroxide value (0.035 millimoles of oxygen per kg fat). On 5th day, all the ghee samples differed significantly ($P<0.05$) from each other in terms of peroxide value (0.054, 0.072 and 0.083 in millimoles of oxygen per kg fat for control, VA2500+FPP25 and VA2500+FG25).

Table 4.55: Effect of storage in glass bottles at 4-7ºC on the peroxide value (millimoles of O_2 per kg fat) of ghee prepared from fortified milk

Storage time→ **Sample↓**	**0 day**	**3rd day**	**5th day**	**7th day**
Control	0.000^{aA}	0.024 ± 0.002^{bA}	0.054 ± 0.004^{cA}	0.084 ± 0.001^{dA}
VA2500+FPP25	0.000^{aA}	0.029 ± 0.001^{bA}	0.072 ± 0.001^{cB}	0.159 ± 0.013^{dB}
VA2500+FG25	0.000^{aA}	0.035 ± 0.001^{bB}	0.083 ± 0.004^{cC}	0.229 ± 0.011^{dC}

Data are presented as means±SEM (n=3).

$^{a\text{-}b}$Means within rows with different lowercase superscript are significantly different ($P<0.05$) from each other.

$^{A\text{-}B}$Means within columns with different uppercase superscript are significantly different ($P<0.05$) from each other.

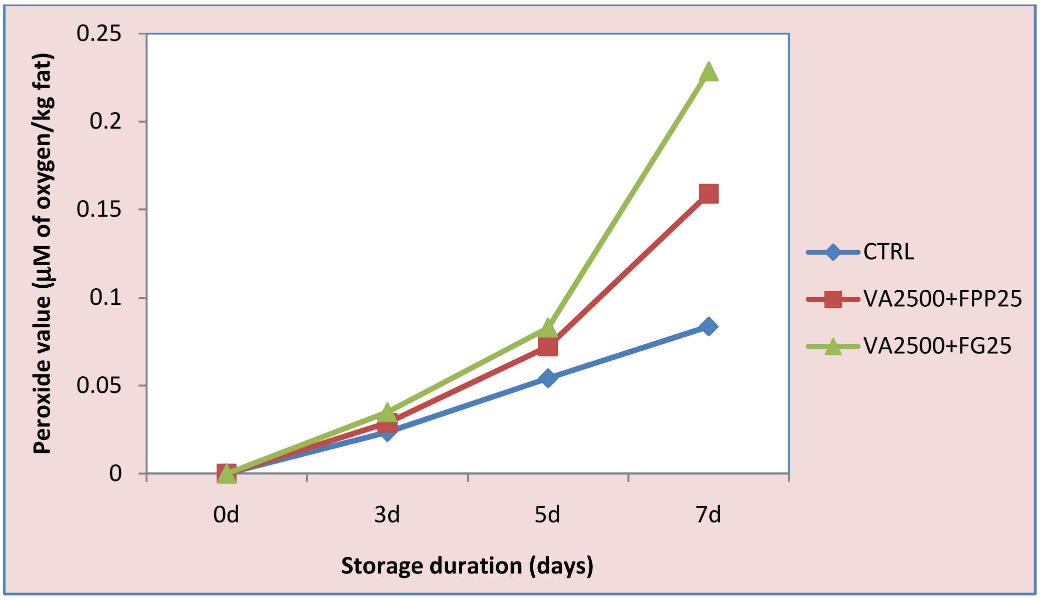

Figure 4.25: Effect of storage in glass bottles at 4-7ºC on the peroxide value of ghee prepared from fortified milk

Our findings were in accordance with the findings of Rao and Ramamurthy (1987) who showed that copper and iron accelerated the autoxidation and influenced the type of carbonyl formed and the rate of development of both peroxides and carbonyls was higher in the case of milk fat fractions added with iron as compared to that found in control. Unnikrishnan and Rao (1977b) also reported an increase in peroxide value of ghee made from cream fortified with iron. The mechanism involved is that the metal ions decompose hydroperoxides to generate new chain reactions which further accelerate the oxidation process.

4.4.5.2 Change in % free fatty acids content during storage

Free fatty acids are formed by action of lipase on triglycerides; which is measured to indicate hydrolytic rancidity.

4.4.5.2.1 Change in % free fatty acids content during storage in polyethylene pouches

There was significant difference ($P<0.05$) in % free fatty acids content of ghee samples even on 3rd day of storage (0.070 on 0 day and 0.083 on 3rd day for control milk; 0.070 on 0 day and 0.090 on 3rd day for VA2500+FPP25; 0.070 on 0 day and 0.097 on 3rd day for FG2500+VA2500) as shown in table 4.56 and figure 4.26. On 0 day, there was no difference in the % free fatty acid content of all the samples. On 3rd day, there was significant difference ($P<0.05$) in % free fatty acids content of all the ghee samples (0.083, 0.090 and 0.097 for control, VA2500+FPP25 and VA2500+FG25, respectively).

Table 4.56: Effect of storage in polyethylene pouches at 4-7ºC on free fatty acids content (%) of ghee prepared from fortified milk

Storage time→ Sample↓	**0 day**	**3rd day**	**5th day**	**7th day**
Control	0.070 ± 0.006^{aA}	0.083 ± 0.007^{bA}	0.11 ± 0.006^{cA}	0.153 ± 0.009^{dA}
VA2500+FPP25	0.070 ± 0.006^{aA}	0.090 ± 0.000^{bB}	0.123 ± 0.007^{cB}	0.167 ± 0.003^{dB}
VA2500+FG25	0.070 ± 0.006^{aA}	0.097 ± 0.003^{bC}	0.133 ± 0.007^{cC}	0.176 ± 0.003^{dC}

Data are presented as means±SEM (n=3).

$^{a-b}$Means within rows with different lowercase superscript are significantly different ($P<0.05$) from each other.

$^{A-B}$Means within columns with different uppercase superscript are significantly different ($P<0.05$) from each other.

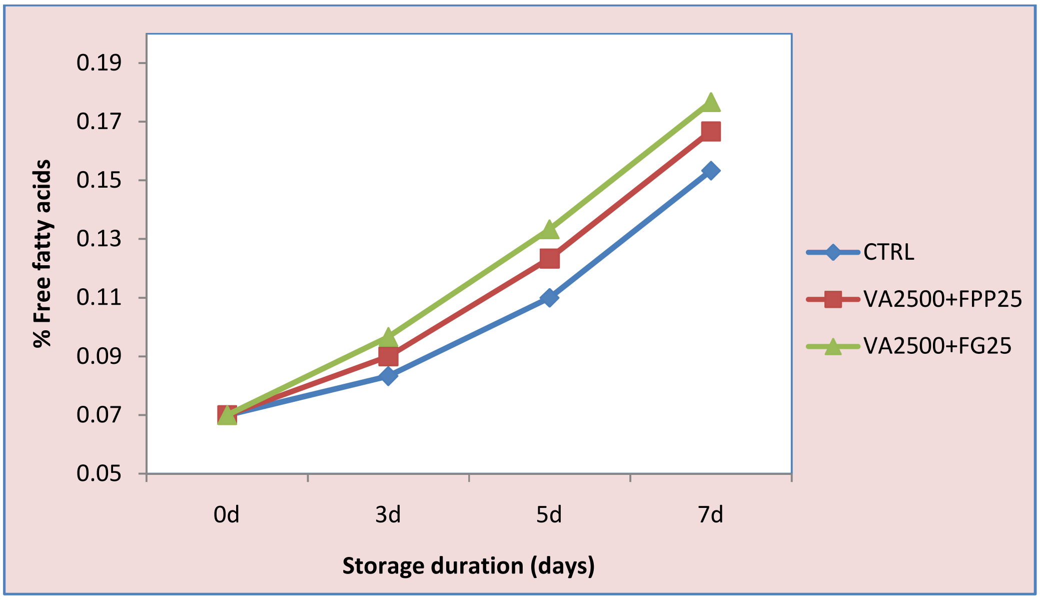

Table 4.26: Effect of storage in polyethylene pouches at 4-7ºC on free fatty acids content (%) of ghee prepared from fortified milk

4.4.5.2.2 Change in % free fatty acids content during storage in glass bottles

It is evident from table 4.57 and figure 4.27 that there was significant difference ($P<0.05$) in % free fatty acid content of ghee samples even on 3rd day of storage (0.063 on 0 day and 0.077 on 3rd day for control; 0.063 on 0 day and 0.083 on 3rd day for VA2500+FPP25; 0.063 on 0 day and 0.09 on 3rd day for FG2500+VA2500). On 0 day, there was no difference in the % free fatty acid content of all the samples.

On 3rd day, % free fatty acid content of control and VA2500+FPP25 did not show any significant difference (0.077 for control milk and 0.083 for VA2500+FPP25), however, VA2500+FG25 differed significantly ($P<0.05$) from control in terms of % free fatty acid content (0.090) but not from VA2500+FPP25. On 5th day, ghee from fortified milk samples differed significantly ($P<0.05$) from control, but not amongst each other in terms of % free fatty acid content (0.103, 0.117 and 0.123 for control, VA2500+FPP25 and VA2500+FG25, respectively). On 7th day, there was significant difference ($P<0.05$) in % free fatty acids content of all the ghee samples (0.143, 0.160 and 0.170 for control, VA2500+FPP25 and VA2500+FG25, respectively).

Table 4.57: Effect of storage in glass bottles at 4-7ºC on the free fatty acids content (%) of ghee prepared from fortified milk

Storage time→ Sample↓	**0 day**	**3rd day**	**5th day**	**7th day**
Control	0.063±0.007^{aA}	0.077±0.009^{bA}	0.103±0.007^{cA}	0.143±0.120^{dA}
VA2500+FPP25	0.063±0.007^{aA}	0.083±0.003^{bAB}	0.117±0.009^{cB}	0.160±0.006^{dB}
VA2500+FG25	0.063±0.007^{aA}	0.090±0.006^{bB}	0.123±0.009^{cB}	0.170±0.006^{dC}

Data are presented as means±SEM (n=3).

$^{a-b}$Means within rows with different lowercase superscript are significantly different (P<0.05) from each other.

$^{A-B}$Means within columns with different uppercase superscript are significantly different (P<0.05) from each other.

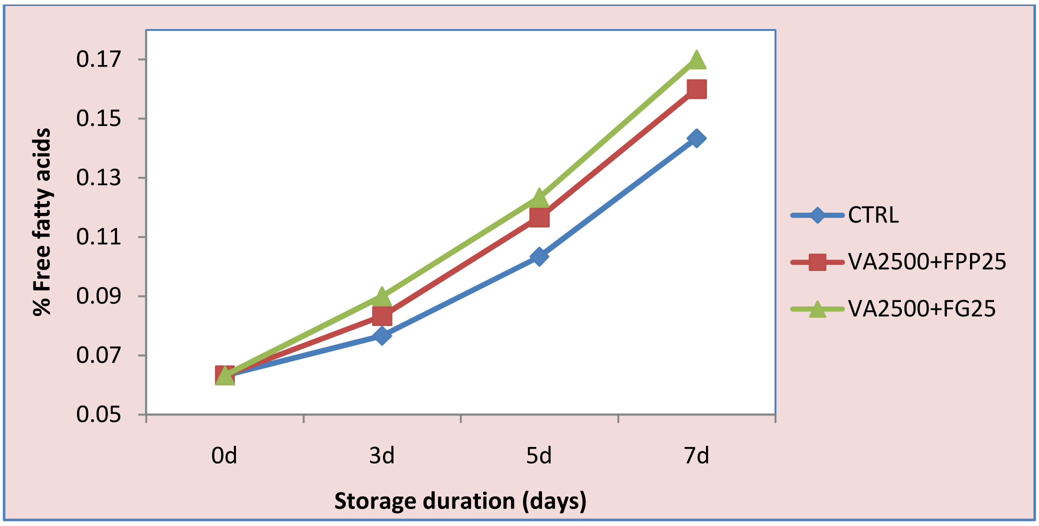

Figure 4.27: Effect of storage in glass bottles at 4-7ºC on the free fatty acids content (%) of ghee prepared from fortified milk

Free fatty acids content (FFA) of normal ghee samples as reported by numerous workers ranged from 0.08 to 1.4% (Kumar 2005). Not much data is available regarding metal ions and % FFA in ghee. Edmonson *et al*. (1971) showed that enrichment of whole milk with ferric iron results in lipolytic rancidity when milk was pasteurised (below 79ºC).

4.4.5.3 Change in TBA value during storage

Thiobarbituric acid (TBA) value is an important fat oxidation parameter. It measures the secondary products of lipid oxidation and it involves reacting thiobarbituric acid with malondialdehyde produced by lipid hydroperoxide decomposition to form a red chromophore with peak absorbance at 532 nm. This coloured complex results in the condensation of two moles of TBA and one mole of malondialdehyde.

4.4.5.3.1 Change in TBA value during storage in polyethylene pouches

It is clear from table 4.58 and figure 4.28 that there was significant difference ($P<0.05$) in TBA value of ghee samples even on 3rd day of storage (0.1437 on 0 day and 0.2253 on 3rd day for control; 0.1439 on 0 day and 0.2447 on 3rd day for VA2500+FPP25; 0.1440 on 0 day and 0.2473 on 3rd day for FG2500+VA2500). On 0 day, there was no difference in TBA value of all the samples. On 3rd day, there was significant difference ($P<0.05$) in TBA value of ghee prepared from control and fortified milk samples but not amongst fortified ghee samples (0.2253, 0.2447 and 0.2473 for control, VA2500+FPP25 and VA2500+FG25, respectively). On 5th day, all the samples differed ($P<0.05$) from each other in terms of TBA value (0.4312, 0.4619 and 0.4729 for control, VA2500+FPP25 and VA2500+FG25, respectively).

Table 4.58: Effect of storage in polyethylene pouches at 4-7ºC on TBA value of ghee prepared from fortified milk

Storage time→ Sample↓	**0 day**	**3rd day**	**5th day**	**7th day**
Control	0.1437 ± 0.003^{aA}	0.2253 ± 0.004^{bA}	0.4312 ± 0.001^{cA}	0.6814 ± 0.004^{dA}
VA2500+FPP25	0.1439 ± 0.003^{aA}	0.2447 ± 0.005^{bB}	0.4619 ± 0.003^{cB}	0.7277 ± 0.001^{dB}
VA2500+FG25	0.1440 ± 0.003^{aA}	0.2473 ± 0.004^{bB}	0.4729 ± 0.005^{cC}	0.7423 ± 0.007^{dC}

Data are presented as means±SEM (n=3).

$^{a-b}$Means within rows with different lowercase superscript are significantly different ($P<0.05$) from each other.

$^{A-B}$Means within columns with different uppercase superscript are significantly different ($P<0.05$) from each other.

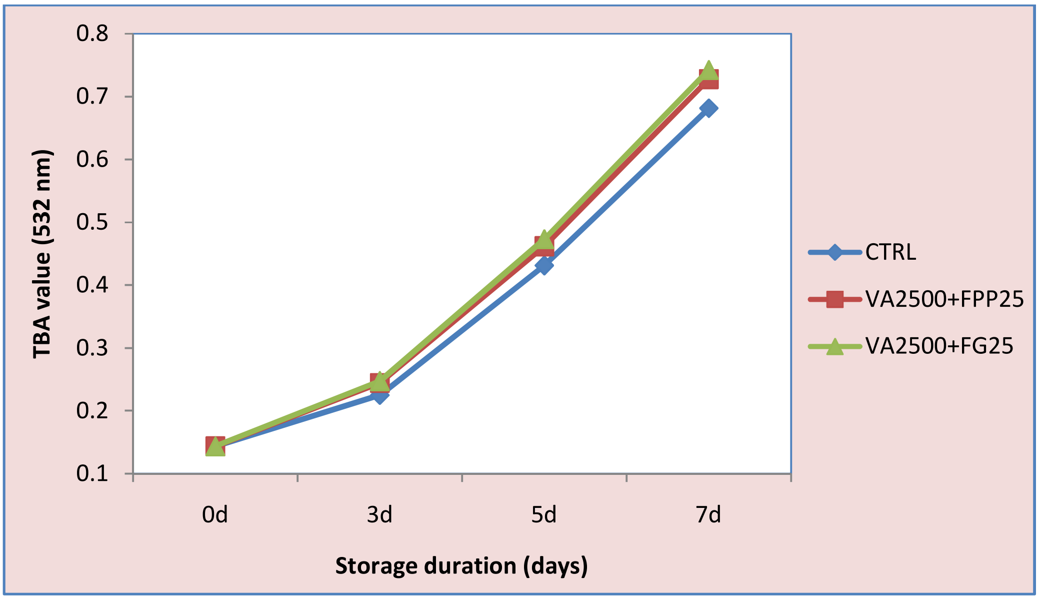

Figure 4.28: Effect of storage in polyethylene pouches at 4-7ºC on TBA value of ghee prepared from fortified milk

4.4.5.3.2 Change in TBA value during storage in glass bottles

It is evident from table 4.59 and figure 4.29 that there was significant difference ($P<0.05$) in TBA value of ghee samples even on 3rd day of storage (0.1445 on 0 day and 0.2270 on 3rd day for control; 0.1446 on 0 day and 0.2438 on 3rd day for VA2500+FPP25; 0.1448 on 0 day and 0.25074 on 3rd day for FG2500+VA2500). On 0 day, there was no difference in TBA value of all the samples. On 3rd day, there was significant difference ($P<0.05$) in TBA value of all ghee samples (0.2270, 0.2438 and 0.2507 for control, VA2500+FPP25 and VA2500+FG25, respectively).

Table 4.59: Effect of storage in glass bottles at 4-7ºC on TBA value of ghee prepared from fortified milk

Storage time→ Sample↓	**0 day**	**3rd day**	**5th day**	**7th day**
Control	0.1445±0.003^{aA}	0.2270±0.005^{bA}	0.4334±0.006^{cA}	0.6646±0.006^{dA}
VA2500+FPP25	0.1446±0.003^{aA}	0.2438±0.005^{bB}	0.4618±0.003^{cB}	0.7245±0.012^{dB}
VA2500+FG25	0.1448±0.003^{aA}	0.2507±0.004^{bC}	0.4716±0.005^{cC}	0.7439±0.009^{dC}

Data are presented as means±SEM (n=3).

$^{a-b}$Means within rows with different lowercase superscript are significantly different ($P<0.05$) from each other.

$^{A-B}$Means within columns with different uppercase superscript are significantly different ($P<0.05$) from each other.

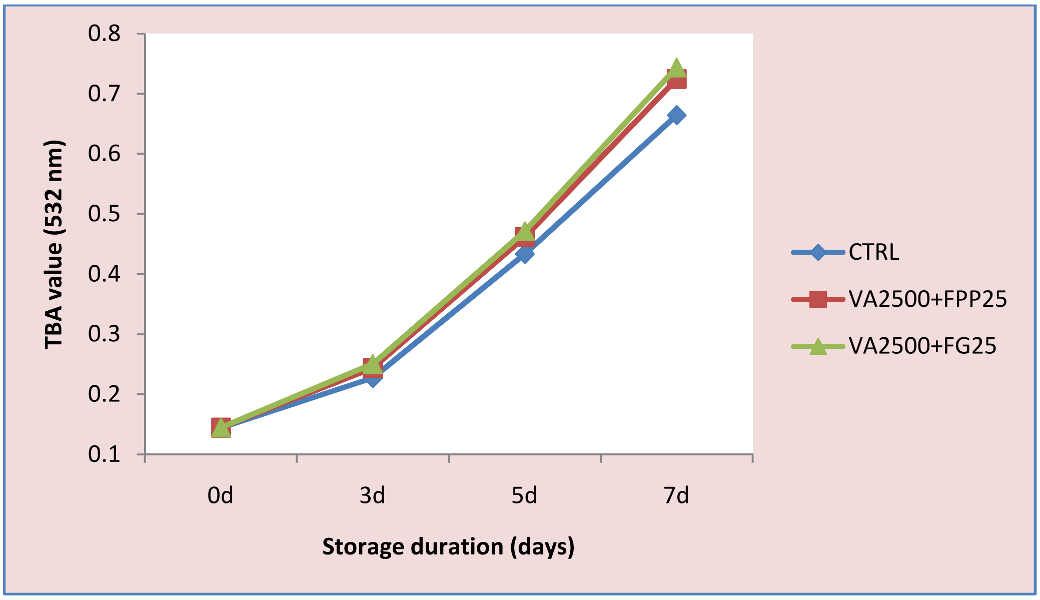

Figure 4.29: Effect of storage in glass bottles at 4-7ºC on TBA value of ghee prepared from fortified milk

Rao and Ramamurthy (1987) reported that the presence of metal catalysts not only accelerates the rate of breakdown of peroxides but also affects the course of reaction during the break down (Lea and Swaboda 1962; Downey 1969). The breakdown of peroxides leads to the accumulation of monocarbonyls directly responsible for the off-flavours in autooxidised milk fat. The breakdown of peroxides can undergo different reactions to give rise to various secondary oxidation products which include different classes of monocarbonyls (Lea and Swaboda 1962).

Downey (1969) reported that metal catalyst accelerates the ratio of breakdown of peroxides and also affects the course of reaction during the break down. Our results were in accordance with Wang and King (1973) who reported four times higher TBA values in ferrous sulphate fortified cow's milk kept for 48 hrs in a cold storage as compared to the ferric ammonium citrate fortified counterpart. Edmonson *et al*. (1971) reported that ferrous sulphate addition at 10 ppm resulted in oxidised flavour, whereas ferric ammonium citrate containing milks developed rancid flavours, but not oxidised.

However, Saini *et al*. (1987) observed that there were negligible differences in the TBA values of 48 and 96 hr stored pasteurised milks fortified with 10 ppm of iron as ferrous and ferric iron with or without ascorbic acid. Heat processing seemed to stabilise the milk against oxidative deterioration, possibly due to SH groups affecting the oxidation-reduction potential of the system. Under certain circumstances, particularly in products with a low protein but high milk fat content, the presence of iron causes oxidative deterioration (Kehagias and Radma 1973). Edmonson *et al*. (1971) have shown that enrichment of whole milk with ferric iron results in lipolytic rancidity when milk was pasteurised (below 79ºC). Guzun-Cojocaru *et al*. (2009) cited that iron incorporated into food systems induces oxidation and precipitation.

4.4.5.4 Change in induction period (oxidative stability index) during storage

The effect of addition of iron and vitamin A on oxidative stability of fat extracted from fortified milk was evaluated by Rancimat equipment. The induction time was used as a major indicator of oxidative stability of fat samples extracted from different types of milk (control and fortified). The induction period (IP) which is also known as oxidative stability index (OSI) is measured as the time required to reach an endpoint of oxidation corresponding to either a level of detectable rancidity or a sudden change in the rate of oxidation (Frankel 1993; Presa-Owens *et al*. 1995). Measurements of induction period under standard conditions were used as an index of oxidative stability of fat.

4.4.5.4.1 Change in induction period (oxidative stability index) during storage in polyethylene pouches

It can be depicted from table 4.60 and figure 4.30 that there was significant difference ($P<0.05$) in induction period (oxidative stability index) of ghee samples even on 3rd day of storage (25.64 on 0 day and 23.81 on 3rd day for control; 23.61 on 0 day and 21.42 on 3rd day for VA2500+FPP25; 22.56 on 0 day and 19.74 on 3rd day for FG2500+VA2500). All the ghee samples differed significantly ($P<0.05$) from each other in terms of induction period (oxidative stability index) even on 0 day (25.64, 23.61 and 22.56 for control, VA2500+FPP25 and VA2500+FG25, respectively).

Table 4.60: Effect of storage in polyethylene pouches at 4-7ºC on induction period (hrs) of ghee prepared from fortified milk

Storage time→ Sample↓	0 day	3rd day	5th day	7th day
Control	25.64±0.06^{aA}	23.81±0.58^{bA}	18.42±0.12^{cA}	15.39±0.06^{dA}
VA2500+FPP25	23.61±0.09^{aB}	21.42±0.04^{bB}	15.24±0.01^{cB}	12.28±0.18^{dB}
VA2500+FG25	22.56±0.10^{aC}	19.74±0.09^{bC}	14.01±0.19^{cC}	11.13±0.08^{dC}

Data are presented as means±SEM (n=3).
$^{a-b}$Means within rows with different lowercase superscript are significantly different (P<0.05) from each other.
$^{A-B}$Means within columns with different uppercase superscript are significantly different (P<0.05) from each other.

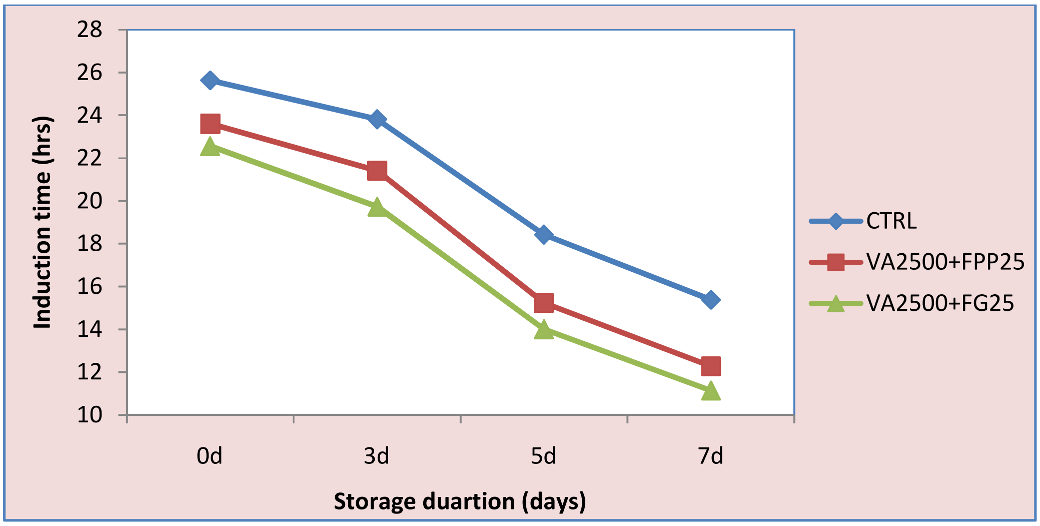

Figure 4.30: Effect of storage in polyethylene pouches at 4-7ºC on induction period (oil stability index) of ghee prepared from fortified milk

4.4.5.4.2 Change in induction period (oxidative stability index) during storage in glass bottles

It is evident from table 4.61 and figure 4.31 that there was a significant difference (P<0.05) in induction period (oxidative stability index) of ghee samples even on 3rd day of storage (26.51 hrs on 0 day and 24.00 hrs on 3rd day for control milk; 23.82 hrs on 0 day and 21.21 hrs on 3rd day for VA2500+FPP25; 22.66 hrs on 0 day and 19.91 hrs on 3rd day for FG2500+VA2500). All the ghee samples differed significantly (P<0.05) from each other in terms of induction period (oxidative stability index) even on 0 day (26.51, 23.82 and 22.66 hrs for control, VA2500+FPP25 and VA2500+FG25, respectively).

Table 4.61: Effect of storage in glass bottles at 4-7ºC on induction period (hrs) of ghee from fortified milk

Storage time→ **Sample↓**	**0 day**	**3rd day**	**5th day**	**7th day**
Control	26.51±0.34^{aA}	24.00±0.27^{bA}	18.79±0.23^{cA}	15.50±0.15^{dA}
VA2500+FPP25	23.82±0.24^{aB}	21.21±0.06^{bB}	15.1±0.07^{cB}	12.55±0.12^{dB}
VA2500+FG25	22.66±0.14^{aC}	19.91±0.06^{bC}	13.61±0.25^{cC}	10.91±0.13^{dC}

Data are presented as means±SEM (n=3).

$^{a-b}$Means within rows with different lowercase superscript are significantly different (P<0.05) from each other.

$^{A-B}$Means within columns with different uppercase superscript are significantly different (P<0.05) from each other.

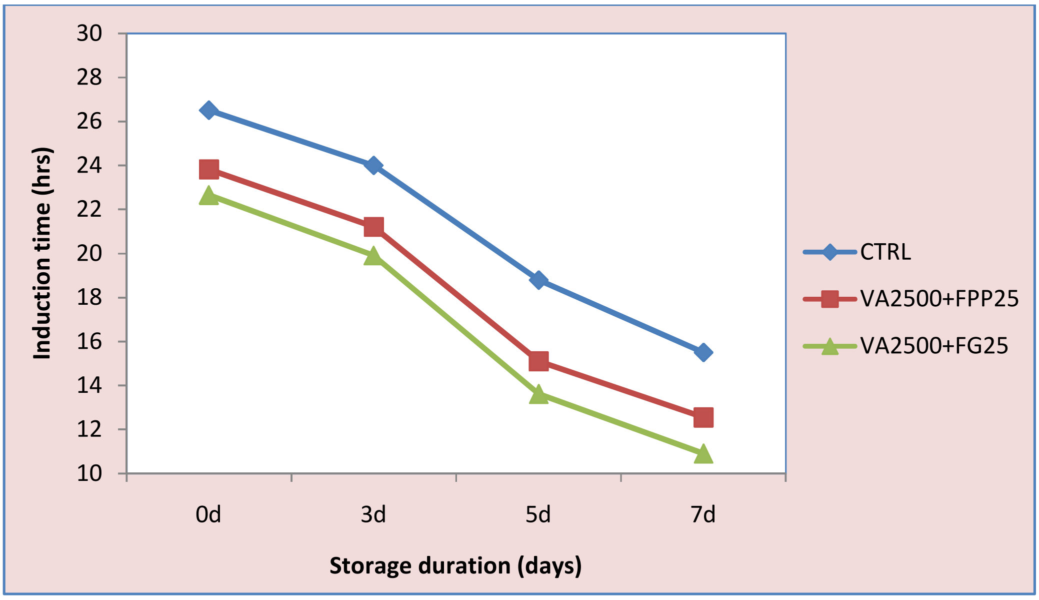

Figure 4.31: Effect of storage in glass bottles at 4-7ºC on induction period (oil stability index) of ghee prepared from fortified milk

Under certain circumstances, particularly in products with a low protein but high milk fat content, the presence of iron causes oxidative deterioration (Kehagias and Radma 1973). Guzun-Cojocaru *et al*. (2009) cited that iron incorporated into food systems induces oxidation and precipitation. This could be the main cause for the reduced induction period of ghee made from cream separated from iron fortified milk. At higher temperature, the formation of volatile secondary products of oxidation occurs, which causes the sudden shoot up in the conductivity and hence the induction period in a shorter period (Gayathry 2011).

It is evidently clear from the results of the study of oxidative stability of milk during storage in polyethylene pouches and glass bottles that the magnitude of difference in peroxide value, % free fatty acids content, TBA value and induction period was higher for ghee from FG hydrate fortified milk as compared to that from FPP soluble fortified milk. This may be due to the fact that FPP soluble is a chelated salt of iron and may interact lesser with the matrix material compared to ferrous gluconate (FG) hydrate which is a highly soluble salt of iron and can interact and affect the milk components more than ferric pyrophosphate (FPP) soluble. Wang and King (1973) reported that the stability of the ferric ammonium citrate (FeAC) fortified milks must at least partially concern the structure of the iron salt itself. FeAC remains largely undissociated or is readily complexed to some component in milk thus inhibiting its lipid oxidative activity. They also reported that the structure of iron salt itself, in part at least, determines its potency in affecting lipid oxidation. Ferrous iron has been shown to be more detrimental than ferric iron causing oxidative deterioration (Mulder *et al.* 1949; Demott 1971; Edmonson *et al.* 1971). It has been demonstrated by Arrington and Krienke (1954) and King and Dunkley (1959) that metal chelators added to milk inhibit metal induced lipid oxidation. Similarly, ferrous ion in ferrous gluconate (FG) hydrate seemed to cause more oxidation, and hence greater loss of vitamin A than ferric ion (chelated with sodium citrate) in ferric pyrophosphate (FPP) soluble. However, no correlation could be drawn related to the comparative oxidative stability of milk stored in polyethylene pouches and glass bottles.

4.5 Effect of fortification on curd tension

Slight increase in curd tension of milk was observed when milk was fortified with vitamin A and iron (table 4.62), however, statistically, the difference was non significant ($P<0.05$) and the values were comparable with the control milk (43.1309, 43.1379 and 43.1382 g for control, VA2500+FPP25 and VA2500+FG25, respectively). There are no reports regarding effect of iron on curd tension of milk. However, our findings could be related to findings of Gaucheron *et al.* (1997a) on curd firmness. They had reported decrease in curd firmness of skim milk fortified with $FeCl_2$ and a slight decrease in case of $FeCl_3$ fortification and suggested that the secondary (aggregation of paracasein micelles) and tertiary (gel formation) phases could be altered by modifications of molecular interactions between paracasein micelles in the presence of iron ions which might have led to slight decrease in curd firmness.

Table 4.62: Effect of vitamin A and iron fortification on curd tension of milk

Sample	Curd tension (g)
Control milk	43.1309±1.26^{a}
VA2500+FPP25	43.1379±1.26^{a}
VA2500+FG25	43.1382±1.26^{a}

Data are presented as means±SEM (n=3).
$^{a-b}$Means with same superscript in column do not vary significantly (P<0.05) from each other.

4.6 Effect of fortification on viscosity

Viscosity of vitamin A+iron fortified milk and control milk was measured at 27ºC using Ostwald's U-tube viscometer and the results are shown in table 4.63. There was non significant effect of fortification (P<0.05) with vitamin A and iron on viscosity of milk and the values were similar with the control milk. There are no reports available regarding the impact on viscosity of addition of iron salts to milk.

However, Gaucheron *et al.* (1997a) reported that there was protein coagulation at 4.0 mM concentration iron, which could possibly affect the viscosity of fortified milk. Dhaka (1982) observed that salts which disperse the casein micelles increase viscosity of milk. Change in the viscosity of milk was not due to change in pH, it was due to the specific property of ions of salts.

Table 4.63: Effect of vitamin A and iron fortification on viscosity of milk at 27ºC

Sample	Absolute viscosity (cP)
Control milk	1.42±0.01^{a}
VA2500+FPP25	1.42±0.01^{a}
VA2500+FG25	1.42±0.01^{a}

Data are presented as means±SEM (n=3).
$^{a-b}$Means with same superscript in column do not vary significantly (P<0.05) from each other.

4.7 Effect of fortification on rennet coagulation time

The rennet coagulation time (RCT) is important to know the amount of rennet and time required during cheese manufacturing. Therefore, RCT of control and fortified milk samples was measured. Coagulation time is determined merely by adding rennet to the milk at a definite temperature and determining the time required for floccules to form. Rennet coagulation times (RCT) of control and vitamin A+iron fortified milk are represented in the table 4.64. Slight increase in rennet coagulation time of milk was observed when milk was fortified with vitamin A and iron, however, statistically, the difference was non significant ($P<0.05$) and the values were comparable with the control (145.67, 161.00 and 167.33 seconds for control, VA2500+FPP25 and VA2500+FG25 fortified milk, respectively). Our findings were in agreement with Reddy and Mahoney (1992) who indicated that ferric chloride at a concentration of 0.2 mM does not significantly affect the rennet clotting time.

Gaucheron *et al*. (1997a) reported that increase in $FeCl_2$ and $FeCl_3$ concentration in skim milk induced: strong increases in clotting times; increases in the aggregation times; and decreases in the curd firmness. In parallel, the initial rates and the total amounts of caseinomacropeptides (CMP) released after rennet addition to supplemented skim milk containing 1.5 mM of iron were not different to those observed with no iron-supplemented skim milk. Thus, the primary phase of rennet coagulation was not affected by the presence of iron. Their results suggested that: (i) chymosin was probably not modified by iron ions; (ii) Ҡ-casein, which has one or two phosphoseryl residues per molecule (Leonil *et al.* 1995) was unmodified; and (iii) the preferential binding of iron to α_{s1}-casein did not affect the enzymatic hydrolysis of Ҡ-casein. Hence, the secondary (aggregation of paracasein micelles) and tertiary (gel reticulation) phases could be altered by modifications of molecular interactions between paracasein micelles in the presence of iron ions. However, Green and Marshall (1977); Marshall and Green (1980); Green (1982) and Pierre (1983) have described acceleration of casein micelle rennet coagulation by divalent cations and polycations. Their interpretation was based on neutralisation of the negative charges of casein micelles and an enhancement of hydrophobic interactions between particles during aggregation and gel reticulation.

Table 4.64: Effect of iron and vitamin A fortification on rennet coagulation time of milk

Sample	Rennet coagulation time (seconds)
Control milk	145.67±9.40^{a}
VA2500+FPP25	161.00±9.54^{a}
VA2500+FG25	167.33±8.41^{a}

Data are presented as means±SEM (n=3).
$^{a-b}$Means with same superscript in column do not vary significantly ($P<0.05$) from each other.

4.8 Effect of fortification on alcohol stability of milk

The alcohol (ethanol) stability test has been used as a simple indicator of milk's freshness and suitability for UHT processing (Shew 1981). Milk should be stable in 74% alcohol to be suitable for UHT treatment (Boumpa *et al*. 2008). Effect of vitamin A and iron salts addition on ethanol stability of milk is shown in table 4.65. All the fortified milk samples did not coagulate on addition of ethanol (75%) i.e. all samples were alcohol negative indicating that the fortified milk had good heat stability and can be used for manufacturing ultrahigh temperature treated dairy products.

Table 4.65: Effect of vitamin A and iron on alcohol stability of milk

Sample	Result
Control milk	Alcohol negative
VA2500+FPP25	Alcohol negative
VA2500+FG25	Alcohol negative

4.9 Effect of fortification on dry sediment (%) in milk

Sediment measurement is considered to be a good indicator of heat stability of a product. Higher the amount of sediment, lesser will be the suitability of milk for high temperature processing. Dry sediment (%) in vitamin A+iron fortified milk was determined by centrifugation of milk (1 ml) at 15000 rpm at 20ºC for 30 minutes. Supernatant was discarded and pellet was dried at 105ºC to constant weight and their % dry sediment was calculated. Slight increase in % dry sediment of milk was observed (table 4.66) when milk was fortified with vitamin A and iron. Statistically, the difference was significant ($P<0.05$), however, the values were comparable with the control milk (2.84, 3.26, 3.39, 3.30 and 3.49 for control, VA2500+FPP20, VA2500+FPP25, VA2500+FG20 and VA2500+FG25 fortified milk, respectively). The low values indicated high level of stability for fortified milk and such milk is considered to be suitable for ultra-high temperature treatment.

Table 4.66: Dry sediment (%) in control and fortified milk

Sample	% Dry sediment
Control milk	2.84±0.05^{a}
VA2500+FPP20	3.26±0.13^{b}
VA2500+FPP25	3.39±0.11^{b}
VA2500+FG20	3.30±0.13^{b}
VA2500+FG25	3.49±0.13^{b}

Data are presented as means±SEM (n=3).
$^{a-b}$Means with same superscript in column do not vary significantly (P<0.05) from each other.

4.10 Effect of fortification on colour profile of milk

Colour is an important criterion while fortifying light coloured foods with iron fortificants. Colour of control and fortified milks was determined by Hunter lab coordinates. The colour measured with colourflex was described in terms of L* value (lightness), a* value (red-green) and b* value (yellow-blue). Colour was recorded after 24 hrs of storage at 4-5ºC. Colourflex readings for control and vitamin A+iron fortified milk are represented in the table 4.67. Fortification of milk with vitamin A and iron did not significantly affect (P<0.05) the colour profile of milk. There was slight but non significant change (P<0.05) in whiteness 'L' value of milk after fortification ('L' value was 83.34, 83.20 and 83.17 for control, VA2500+FPP25 and VA2500+FG25, respectively). ΔL value for VA2500+FPP25 and VA2500+FG25 was 0.14 and 0.18, respectively, showing slight increase in darkness of milk samples after fortification i.e. darkening was more in case of FG compared to FPP. Similarly, 'a' value i.e. greenness (-) ('a' value was -1.48, -1.58 and -1.61 for control, VA2500+FPP25 and VA2500+FG25, respectively) and 'b' value i.e. yellowness (+) ('b' value was 6.83, 6.93 and 7.01 for control, VA2500+FPP25 and VA2500+FG25 fortified milk samples, respectively) also did not show any significant difference (P<0.05) between control and both types of fortified milks. In present findings, there was a change with regard to greenness and yellowness. However, Douglas *et al*. (1981) reported darkening of chocolate milk fortified with FeAC and FPP at 10 ppm level and suggested that the difference observed could be due to the difference in original colour of milk and chocolate milk.

Table 4.67: Effect of vitamin A and iron fortification on colour profile of milk

Sample	L	∆L	a	∆a	b	∆b
Control milk	83.34±0.20^a	-	1.48±0.07^a	-	6.83±0.23^a	-
VA2500+FPP25	83.20±0.20^a	0.14±0.02	1.58±0.06^a	0.10±0.01	6.93±0.23^a	0.10±0.01
VA2500+FG25	83.17±0.20^a	0.18±0.01	1.61±0.06^a	0.13±0.01	7.01±0.18^a	0.18±0.05

Data are presented as means±SEM (n=3).
$^{a-b}$Means with same superscript in column do not vary significantly ($P<0.05$) from each other.

4.11 Distribution of iron among different milk fractions

Distribution of added iron salts into major milk fractions was studied. Whole milk was fortified with FPP soluble and FG hydrate (@25 ppm iron) and was separated into different fractions viz. skim milk, ghee, ghee residue, casein and whey and % distribution of iron was determined into these milk fractions.

It is clear from table 4.68 that in control milk 87.44% iron was in skim milk, 2.51% in ghee and 10.05% in ghee residue. Out of the iron present in skim milk (87.44%), 64.32% was bound to casein and 23.12% was in whey (table 4.69 and figure 4.32).

In case of FPP soluble fortified milk, 86.67% iron was in skim milk, 2.42% was in ghee and 10.90% was in ghee residue (table 4.68). Out of the iron present in skim milk (86.67%), 64.82% was bound to casein and 21.85% was in whey fraction (table 4.69 and figure 4.33).

In case of FG hydrate fortified milk, 86.98% iron was in skim milk, 2.46% in ghee and 10.56% in ghee residue (table 4.68). Out of the iron present in skim milk (86.98%), 64.56% was bound to casein and 22.42% was in whey fraction (table 4.69 and figure 4.34).

Table 4.68: Distribution of iron among different milk fractions

Milk fraction	Conc. in ctrl milk (ppm)	% Distribution	Conc. in FPP fortd milk (ppm)	% Distribution	Conc. in FG fortd milk (ppm)	% Distribution
Whole milk	1.154 ±0.065	-	25.860 ±0.068	-	25.882 ±0.098	-
Skim milk	0.920 ±0.093	87.44 ±0.52	21.249 ±1.137	86.67 ±0.81	21.345 ±1.110	86.98 ±0.73
Ghee	1.109 ±0.043	2.51 ±0.09	24.101 ±0.907	2.42 ±0.09	24.485 ±0.823	2.46 ±0.09
Ghee residue	22.621 ±0.341	10.05 ±0.44	552.725 ±35.701	10.90 ±0.73	535.690 ±31.576	10.56 ±0.66

Data are presented as means±SEM (n=3).

Table 4.69: Distribution of iron between casein and whey

Milk fraction	Conc. in ctrl milk (ppm)	% Distribution	Conc. in FPP fortd milk (ppm)	% Distribution	Conc. in FG fortd milk (ppm)	% Distribution
Skim milk	0.920 ±0.093	87.44 ±0.52	21.249 ±1.137	86.67 ±0.81	21.345 ±1.110	86.98 ±0.73
Casein	5.303 ±0.323	64.32 ±0.31	119.364 ±1.019	64.82 ±0.22	119.350 ±0.752	64.56 ±0.27
Whey	0.267 ±0.018	23.12 ±0.24	5.652 ±0.161	21.85 ±0.59	5.804 ±0.162	22.42 ±0.55

Data are presented as means±SEM (n = 3).

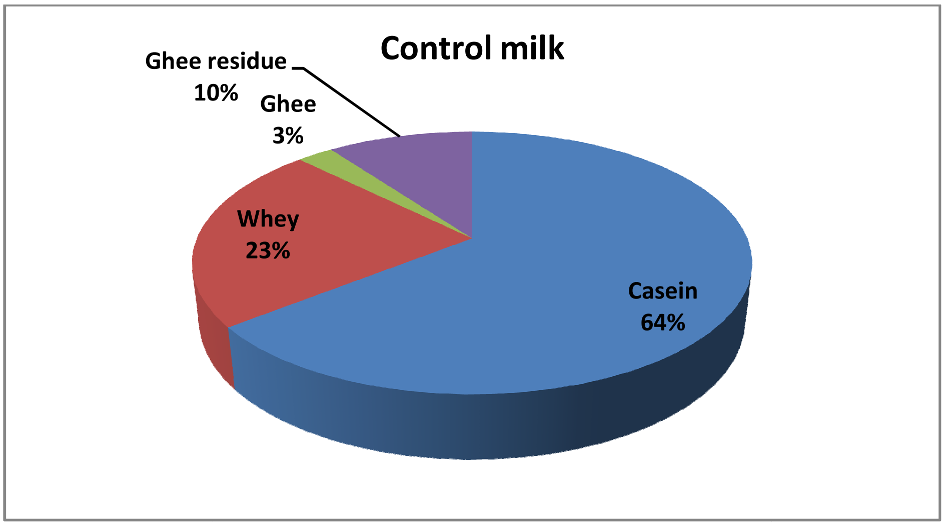

Figure 4.32: Distribution of iron in different fractions in control milk

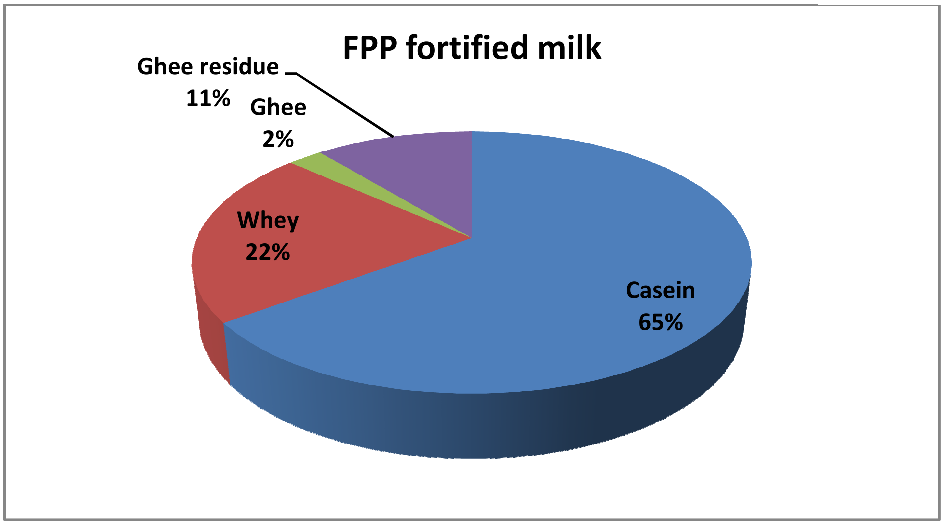

Figure 4.33: Distribution of iron in different fractions in FPP soluble fortified milk (@25 ppm iron)

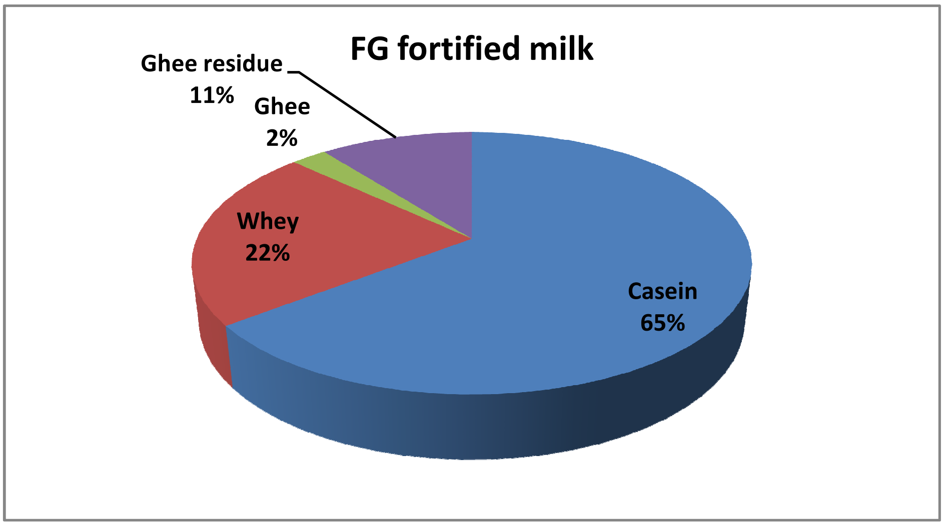

Figure 4.34: Distribution of iron in different fractions in FG hydrate fortified milk (@25 ppm iron)

Our findings were in agreement with Unnikrishnan and Rao (1977a), who reported that around 90% of added iron was associated with skim milk, 71.9% with casein and 19.5% with isoelectric whey. Demott and Park (1973) also reported that 87.28% added iron was associated with skim milk, 67.84% with isoelectric casein and 16.50% with isoelectric whey. This might be due to washing of iron from casein at isoelectric pH (Unnikrishnan and Rao 1977a).

Demott and Dincer (1976) reported that iron, when added as $FeCl_2$ to skim milk, 85% is bound to caseins (72, 21 and 4% are bound to α_{s}-, β and κ-caseins, respectively). King *et al*. (1959) reported that naturally occurring iron was concentrated at the surface of fat globules, but none of the added iron became associated with the fat globules. Natural iron not associated with the fat globules was more concentrated with the whey proteins than with casein. Allan (1950) concluded that both added and natural iron was non-ionic and was associated with the fat globule membrane. The results showed that essentially all of the added iron was associated with the skim milk. The washed creams had only negligible activities. Natural iron was highly concentrated at the fat globule membrane and it favoured the whey proteins over casein.

The strong affinity of caseins for iron is recognised and attributable to clustered phosphoseryl residues (Manson and Cannon 1978; Hegenauer *et al*. 1979b; Gaucheron *et al.* 1995). Higher percentage of iron binding to casein compared to whey proteins can be related to the work of Sugiarto *et al*. (2009) who demonstrated that sodium caseinate had more sites than whey protein isolates to bind iron and that the affinity of caseinate to bind iron was higher than that of whey proteins. This was expected because caseins are known to have a stronger binding capacity than whey proteins for metal cations, mainly because of the presence of clustered phosphoserine residues. The more compact structure of whey proteins (i.e. globular proteins) in whey protein isolate (WPI), compared with the more flexible, open structure of sodium caseinate, could also account for the differences in their ability to bind added iron.

Silva *et al.* (2001) investigated the behaviour of iron in the iron supplemented complex system formed by milk components and inferred that iron is not associated with micellar calcium phosphate. The strong affinity of casein for iron is recognised and attributed to clustered phosphoseryl residues (Gaucheron *et al.* 1997b). The distinct casein fractions are phosphorylated to different degrees. In iron fortified milk, caseins are the principal sequestrant of added iron (Hegenauer *et al.* 1979d; King *et al.* 1959). The oxidative state of iron (Fe^{2+} or Fe^{3+}) does not affect its distribution in milk, but the particular counter-ion in the iron salt seems to have an impact.

4.12 Fractionation of iron in colloidal and soluble phases

Milk was ultracentrifuged in order to separate it into colloidal and soluble phases. In case of control milk, 88.35% iron was in colloidal phase and 11.65% was in soluble phase (table 4.70 and figure 4.35). In case of FPP soluble fortified milk samples, 90.71% iron was present in colloidal phase and 9.29% in soluble phase (table 4.70 and figure 4.36); in FG hydrate fortified milk, 89.81% iron was in colloidal phase and 10.19% iron was in soluble phase (table 4.70 and figure 4.37). Our results were in corroboration with Gaucheron *et al*. (1997a) who reported that iron when added to milk in the concentration range of 0 to 1.5 mM, 89% and more of iron was bound to the colloidal phase. The proportionality between the binding of iron to the colloidal phase and the added concentration suggests that the binding sites were not saturated and had the same affinity for iron ions in the iron concentration range used.

Raouche *et al*. (2009) reported that about 80-90% of iron bound to the colloidal phase and micellar to total iron ratio was 84-95%. Micellar iron could be directly linked to casein and/or to micellar salt, the colloidal calcium phosphate. However, relative quantification of iron after milk ultracentrifugation did not allow distinction between either of the micellar iron forms. Similar findings have been reported by Hegenauer *et al*. (1979d); Gaucheron *et al*. (1997a); Hekmat and McMahon (1998); Gaucheron (2000) and Philippe *et al*. (2005).

Table 4.70: Fractionation of iron in colloidal and soluble phases (ultracentrifugation)

Sample	Iron content (ppm)	Iron in ultracentrifugal supernatant (ppm)	% iron in colloidal phase	% iron in soluble phase
Control milk	1.057±0.091	0.123±0.010	88.35±0.11	11.65±0.11
FPP@25ppm	25.963±0.073	2.412±0.019	90.71±0.07	9.29±0.07
FG@25ppm	25.979±0.094	2.648±0.030	89.81±0.11	10.19±0.11

Data are presented as means±SEM (n=3).

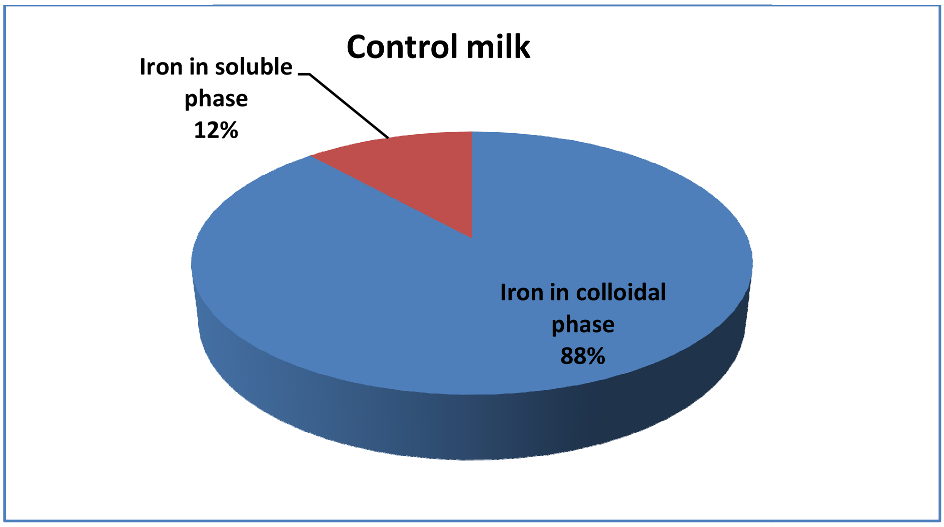

Figure 4.35: Fractionation of iron in colloidal and soluble phases in control milk

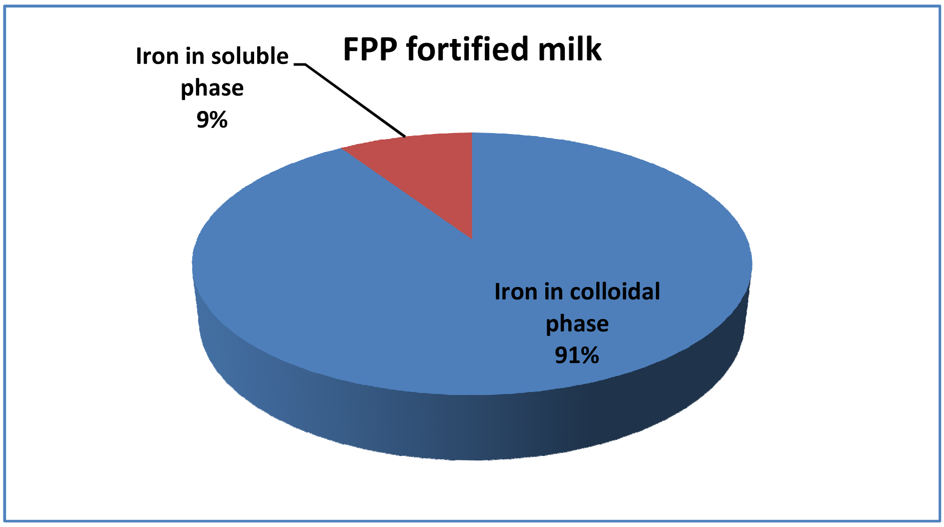

Figure 4.36: Fractionation of iron in colloidal and soluble phases in FPP soluble fortified milk (@25 ppm iron)

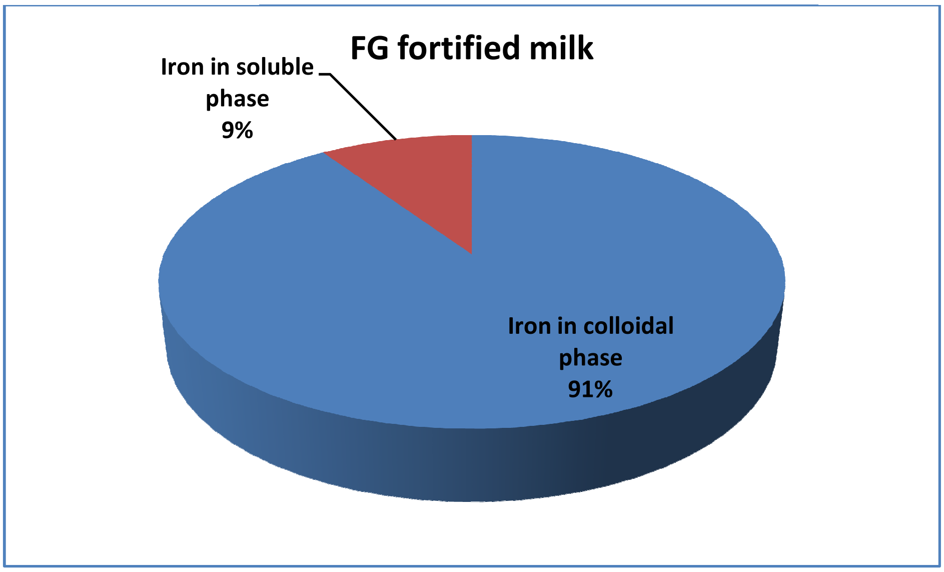

Figure 4.37: Fractionation of iron in colloidal and soluble phases in FG hydrate fortified milk (@25 ppm iron)

4.13 Effect of iron fortification on electrophoretic pattern of milk proteins

With a view to gain knowledge about the electrophoretic behaviour of proteins in milk fortified with selected iron salts (FPP soluble, FG hydrate) and vitamin A acetate; and to investigate the interactive effect of iron salts and vitamin A with milk proteins, the samples were characterised on urea-PAGE and SDS-PAGE

4.13.1 Urea-PAGE

Urea-PAGE methods have been used for the study of milk proteins in which dissociating agent (urea) prevents molecular aggregation. Gel images were processed by means of gel analysis software (Spectronics Corporation ImageAide for Windows) and the densitograms were then drawn. Figure 4.38 depicts the electrophoretic patterns of proteins of control and iron fortified milk. Lane 1 represents purified casein, lane 2 represents control milk proteins, Lane 3, 4, 5 and 6 represent proteins of milk fortified with vitamin A acetate@2500 IU/L+FPP soluble@20 ppm (VA2500+FPP20), vitamin A acetate@2500 IU/L+FPP soluble@25 ppm (VA2500+FPP25), vitamin A acetate@2500 IU/L+FG hydrate@20 ppm (VA2500+FG20) and vitamin A acetate@2500 IU/L+FG hydrate@25 ppm (VA2500+FG25), respectively. Casein in the samples were resolved into two major bands (β-CN and α-CN) which was confirmed by comparison of the band pattern with purified casein (lane 1), two other prominent bands were also obtained which were probably whey proteins viz. β-lactoglobulin (β-lb) and α-lactalbumin (α-la). The height and area under the curve (raw volume) of each lane of urea-PAGE densitogram was measured by gel analysis software ImageAide as represented in table 4.71 and 4.72.

From the tables, it can be concluded that electrophoretic pattern of proteins of control and iron fortified milk did not show any difference in band pattern i.e. there was no difference in mobility based on charge of the proteins; the intensity (height) and raw volume of bands also did not show any significant difference ($P<0.05$). This indicated that selected iron salts (upto 25 ppm iron) and vitamin A acetate (2500 IU/L) could be added to milk system without significantly affecting the electrophoretic behaviour. Our results were in agreement with Ravikiran *et al*. (1977) who reported that fortification of milk with ferric lactose did not alter the fractionation behaviour of milk proteins nor the electrophoretic mobility of the proteins either in the buffalo or cow milk.

Table 4.71: ImageAide readings of height of different bands of each lane of urea-PAGE gel

Sample→ Band no.↓	**Control Lane 2**	**VA2500+ FPP20 Lane 3**	**VA2500+ FPP25 Lane 4**	**VA2500+ FG20 Lane 5**	**VA2500+ FG25 Lane 6**
1	27.04 ± 1.23^{a}	26.54 ± 1.08^{a}	26.01 ± 1.01^{a}	26.16 ± 0.99^{a}	25.94 ± 1.01^{a}
2	55.33 ± 0.61^{a}	55.01 ± 0.63^{a}	54.29 ± 0.59^{a}	54.85 ± 0.65^{a}	54.17 ± 0.57^{a}
3	49.01 ± 0.41^{a}	49.26 ± 0.46^{a}	49.73 ± 0.51^{a}	49.39 ± 0.46^{a}	49.90 ± 0.53^{a}
4	26.11 ± 0.93^{a}	26.48 ± 0.80^{a}	26.79 ± 0.87^{a}	26.56 ± 0.83^{a}	27.02 ± 0.87^{a}

Data are presented as means±SEM (n=3).
$^{a-b}$Means with same superscript in row do not vary significantly ($P<0.05$) from each other.

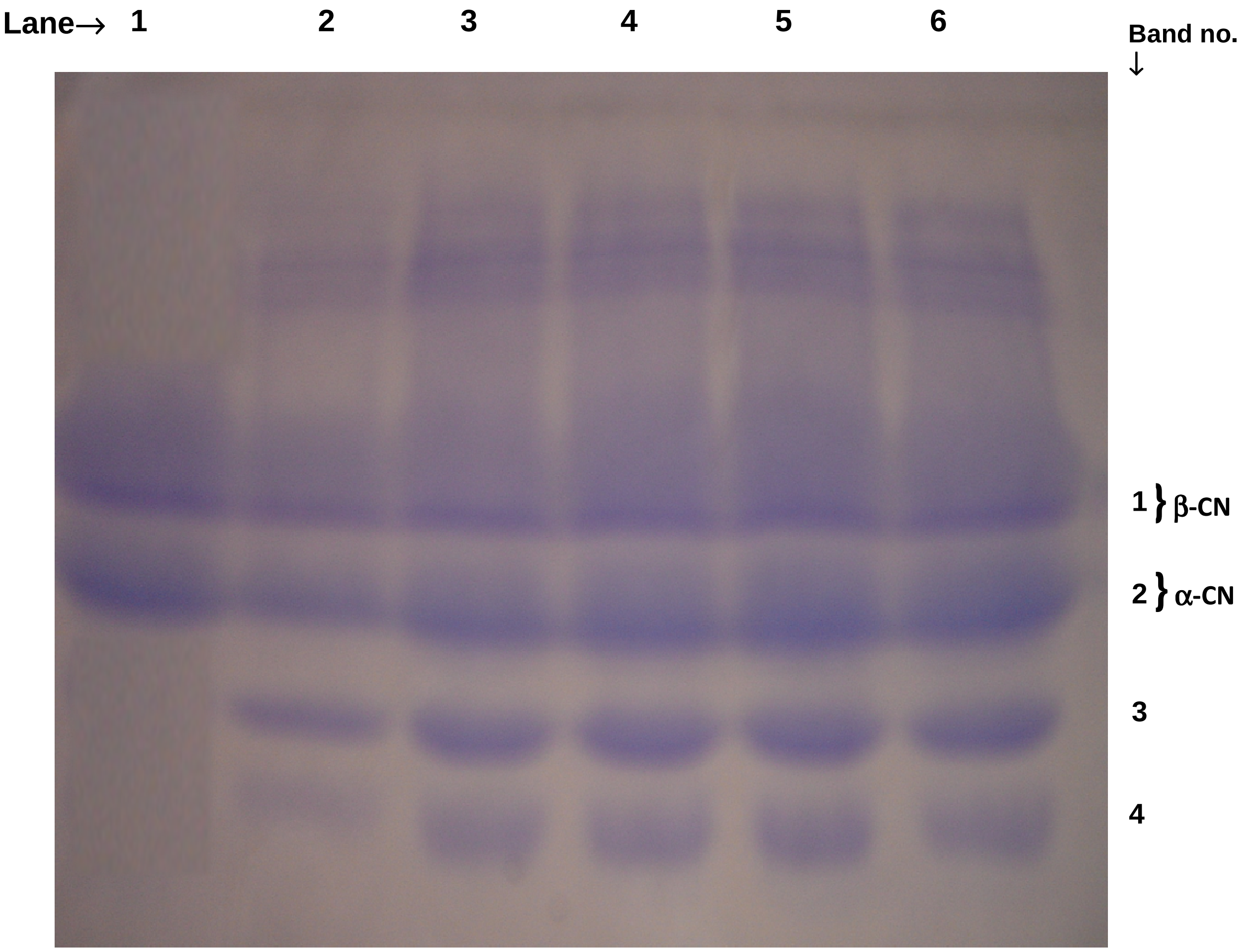

Figure 4.38 Urea-PAGE pattern of milk proteins as affected by vitamin A and iron supplementation separated on 14% gel

Lane 1 Casein

Lane 2 Control milk

Lane 3 Vitamin A acetate@2500 IU/L+FPP soluble fortified milk (@20 ppm iron)

Lane 4 Vitamin A acetate@2500 IU/L+FPP soluble fortified milk (@25 ppm iron)

Lane 5 Vitamin A acetate@2500 IU/L+FG hydrate fortified milk (@20 ppm iron)

Lane 6 Vitamin A acetate@2500 IU/L+FG hydrate fortified milk (@25 ppm iron)

Table 4.72: ImageAide readings of raw volume (area under the curve) of different bands of each lanes of urea-PAGE gel

Sample→ Band no.↓	Control Lane 2	VA2500+ FPP20 Lane 3	VA2500+ FPP25 Lane 4	VA2500+ FG20 Lane 5	VA2500+ FG25 Lane 6
1	501453.07 ±46554.66^{a}	492559.67 ±47586.61^{a}	485389.40 ±46754.99^{a}	487277.57 ±46791.77^{a}	477317.03 ±46763.15^{a}
2	1091058.70 ±73152.24^{a}	1076131.40 ±60942.54^{a}	1035451.20 ±26837.75^{a}	1059642.70 ±47958.43^{a}	1015735.00 ±15523.06^{a}
3	1111515.20 ±25749.81^{a}	1129471.20 ±19147.05^{a}	1147784 ±16487.93^{a}	1194171.00 ±18969.55^{a}	1147419.20 ±14382.37^{a}
4	482322.37 ±8059.18^{a}	484376.58 ±8460.57^{a}	491206.27 ±8861.75^{a}	4872222.86 ±8143.91^{a}	495224.73 ±9412.71^{a}

Data are presented as means±SEM (n=3).
$^{a-b}$Means with same superscript in row do not vary significantly (P<0.05) from each other.

4.13.2 SDS-PAGE

SDS-PAGE was performed for the molecular weight determination. Samples were resolved in 14% SDS gel. The figure 4.39 depicts the electrophoretic patterns of proteins of control and vitamin A+iron fortified milk. Lane 1 represents molecular weight marker (ranging from 205 to 3.5 KDa). Lane 2 represents control milk proteins, Lane 3, 4, 5 and 6 represent proteins of milk fortified with vitamin A acetate@2500 IU/L+FPP soluble@20 ppm (VA2500+FPP20), vitamin A acetate@2500 IU/L+FPP soluble@25 ppm (VA2500+FPP25), vitamin A acetate@2500 IU/L+FG hydrate@20 ppm (VA2500+FG20) and vitamin A acetate@2500 IU/L+FG hydrate@25 ppm (VA2500+FG25), respectively. Milk proteins were resolved in four major bands which were assigned their respective labels (β-CN, α-CN, β-lg and α-la) based on comparison of their molecular weight with molecular weight markers (lane 1). The molecular weight, height and area under the curve (raw volume) of each lane of SDS-PAGE densitogram were measured by gel analysis software ImageAide as represented in table 4.73, 4.74 and 4.75.

From the tables, it can be inferred that protein patterns were unchanged by the presence of vitamin A and iron salts since electrophoretic pattern of proteins of control and vitamin A+iron fortified milk did not show any difference in band pattern i.e. there was no difference in mobility based on size of the proteins; also the intensity (height) and raw volume of bands did not show any significant difference (P<0.05). This indicated that selected iron salts (upto 25 ppm iron) and vitamin A acetate (2500 IU/L) could be added to milk system without significantly affecting the electrophoretic behaviour. Our findings were in agreement with Ravikiran *et al*. (1977) who reported that fortification of milk with ferric lactose did not alter the fractionation behaviour of milk proteins nor the electrophoretic mobility of the proteins either in the buffalo or cow milk.

Table 4.73: ImageAide readings of molecular weight of different bands of each lane of SDS-PAGE gel

Sample→ Band no.↓	**Control Lane 2**	**VA2500+ FPP20 Lane 3**	**VA2500+ FPP25 Lane 4**	**VA2500+ FG20 Lane 5**	**VA2500+ FG25 Lane 6**
1	34226.1 ±649.7^{a}	34234.3 ±650.7^{a}	34108.4 ±653.2^{a}	34160.2 ±632.2^{a}	34289.1 ±383.6^{a}
2	28687.7 ±767.9^{a}	28527.4 ±738.8^{a}	28435.8 ±752.6^{a}	28479.7 ±740.5^{a}	28385.8 ±739.01^{a}
3	18363.4 ±80.4^{a}	18248.3 ±111.8^{a}	18182.1 ±103.3^{a}	18307.0 ±95.2^{a}	18201.0 ±51.9^{a}
4	14387.5 ±77.7^{a}	14428.9 ±90.4^{a}	14490.0 ±98.3^{a}	14452.2 ±98.4^{a}	14530.8 ±88.6^{a}

Data are presented as means ± SEM (n=3).
$^{a-b}$Means with same superscript in row do not vary significantly (P<0.05) from each other.

Table 4.74: ImageAide readings of height of different bands of each lane of SDS-PAGE gel

Sample→ Band no.↓	**Control Lane 2**	**VA2500+ FPP20 Lane 3**	**VA2500+ FPP25 Lane 4**	**VA2500+ FG20 Lane 5**	**VA2500+ FG25 Lane 6**
1	20.55±0.57^{a}	20.21±0.49^{a}	19.95±0.51^{a}	19.98±0.55^{a}	19.77±0.57^{a}
2	20.17±0.93^{a}	20.77±0.60^{a}	20.85±0.59^{a}	20.81±0.69^{a}	20.88±0.70^{a}
3	51.99±0.40^{a}	52.27±0.37^{a}	52.50±0.38^{a}	52.38±0.38^{a}	52.51±0.37^{a}
4	50.43±0.49^{a}	50.66±0.54^{a}	50.99±0.49^{a}	50.77±0.53^{a}	51.01±0.56^{a}

Data are presented as means ± SEM (n=3).
$^{a-b}$Means with same superscript in row do not vary significantly (P<0.05) from each other.

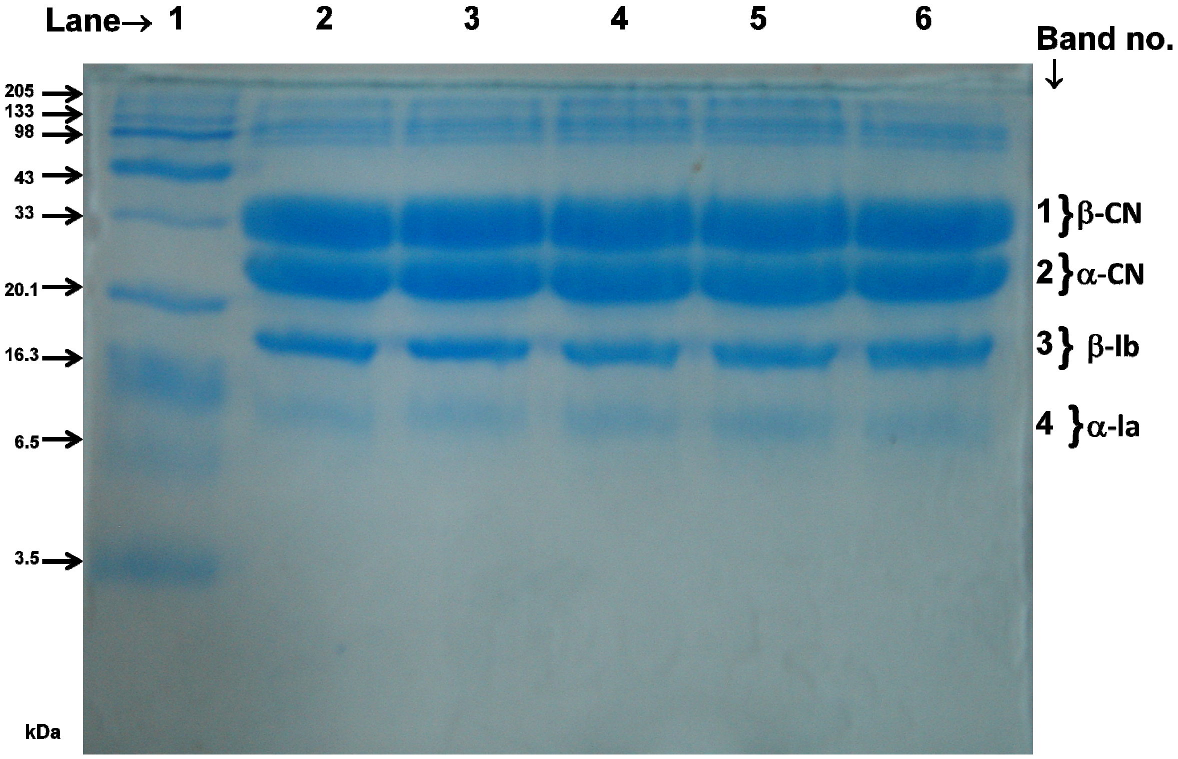

Figure 4.39 SDS-PAGE pattern of milk proteins as affected by iron supplementation separated on 14% gel

Lane 1 Molecular weight markers ranging from 205 kDa to 3.5 kDa

Lane 2 Control milk

Lane 3 Vitamin A acetate@2500 IU/L+FPP soluble fortified milk (@20 ppm iron)

Lane 4 Vitamin A acetate@2500 IU/L+FPP soluble fortified milk (@25 ppm iron)

Lane 5 Vitamin A acetate@2500 IU/L+FG hydrate fortified milk (@20 ppm iron)

Lane 6 Vitamin A acetate@2500 IU/L+FG hydrate fortified milk (@25 ppm iron)

Table 4.75: ImageAide readings of raw volume (area under the curve) of different bands of each lane of SDS-PAGE gel

Sample→ Band no.↓	Control Lane 2	VA2500+ FPP20 Lane 3	VA2500+ FPP25 Lane 4	VA2500+ FG20 Lane 5	VA2500+ FG25 Lane 6
1	227096.20 ±5825.12^{a}	226058.23 ±5830.02^{a}	225172.92 ±6001.56^{a}	225530.70 ±6041.58^{a}	223976.90 ±6298.91^{a}
2	449008.00 ±5868.85^{a}	449359.05 ±5884.51^{a}	449611.00 ±5938.27^{a}	449350.70 ±5817.98^{a}	449984.00 ±6018.58^{a}
3	2109967.00 ±60922.69^{a}	2130325.00 ±70272.56^{a}	2159080.30 ±55851.56^{a}	2151309.00 ±55653.38^{a}	2170646 ±62252.17^{a}
4	1287011.00 ±52217.82^{a}	1288870.40 ±52113.93^{a}	1290478.30 ±51672.81^{a}	1289572.00 ±51766.32^{a}	1294562.00 ±49573.94^{a}

Data are presented as means±SEM (n=3).
$^{a-b}$Means with same superscript in row do not vary significantly (P<0.05) from each other.

4.14 Effect of fortification on Maillard browning

Metal ions are known to activate Maillard reaction (Kato *et al*. 1981), particularly the formation of carboxymethyllysine (Ahmed *et al*. 1986). Furthermore, iron is strongly chelated with glycated proteins (Qian *et al.* 1998) and can generate oxygen species, efficiently reacting with the amino acid residues located near the metal binding site (Amici *et al.* 1989; Stadtman and Oliver 1991). Iron by itself has been reported to accelerate the formation of advanced Maillard products (AMP) from the Amadori product (Kato *et al*. 1981). Iron is potential activator of Amadori product (Wolff *et al*. 1988) autoxidation, the reaction is highly temperature dependent.

Consequently, peroxide and superoxide radicals may be formed and released because of the partial destruction of the milk peroxidase and catalase activities during heating. Therefore, all the reactants of the Haber Weiss-Fenton reaction are present, suggesting that the very reactive hydroxyl radical, which attacks the sensitive sulphur and aromatic amino acids (Gardner 1979) could be produced. However, the Maillard reaction could also be activated by the high lactose content and iron contents (Kato *et al.* 1981) and this reaction can also produce oxygen radical species (Gillery *et al.* 1988). Many of these reactions are oxygen dependent and the very low oxygen concentration in milk is probably a limiting factor, however, some of them have been reported to develop in the absence of oxygen (Yim *et al*. 1995).

Iron (II) rapidly oxidises to iron (III) at neutral pH. Vitamin C and the Amadori product (Wolff *et al*. 1988; Gillery 1988) are potent reducers of iron (III); and hydrogen peroxide and iron (II) are produced in the presence of traces of oxygen, which catalyse the Fenton reaction. Maillard browning also releases free radicals (Hayashi and Namiki 1981; Yim *et al*. 1995). Hence, protein glycation should favour prooxidant conditions. On the other hand, metal ions (Kato *et al.* 1981) and hydroxyl radicals (Kawakishi *et al*. 1990) have also been reported to activate the Maillard browning reaction, particularly the advanced stage of this reaction. They probably act by increasing the conversion rate of the Amadori product to the dicarbonyl compounds (deoxysones) which in turn mediate protein cross linking (Chace *et al.* 1991) or fragmentation (Kawakishi *et al*. 1990).

Monitoring the nutritional quality of dietetic milk throughout its shelf life is particularly important due to the high susceptibility of some vitamins to oxidation and the continuous development of the Maillard reaction during storage (Gliguem and Birlouez-Aragon 2004).

Maillard reaction leads to a decrease in the nutritional value of proteins and a modification in the organoleptic properties of milk (cooked taste and browning). The specific composition of fortified formulas explains their high susceptibility to formation of Maillard products (Birlouez-Aragon *et al*. 1997; Evangelisti *et al.* 1999), especially, the high lactose content, the presence of proteins with many reactive lysine residues (Finot *et al*. 1981) and activation allowed by ferrous ions (Birlouez-Aragon *et al.* 1997).

The reactions described above are complicated by the addition of iron and vitamin C to the fortified milks (Almaas *et al*. 1997). Several useful indicators can be analysed to characterise the consequence of heat treatment on the nutritional quality of heat-treated milks. Lactulosyllysine is the main chemical form of lysine blockage by the Maillard reaction in heat treated milk (Bujard and Finot 1978; Finot *et al.*

1981; Rerat *et al*. 2002), with furosine being a good indicator of the lactulosyllysine content (Resmini and Pellegrino 1991; Pizzoferrato *et al*. 1998; Evangelisti *et al*. 1999). Milk is particularly prone to the Maillard reaction due to its high lactose and lysine content. Fortification with iron, as in case of fortified milk, further accelerates the reaction. Therefore, our aim was to evaluate the effect of different heat treatments on indicator of Maillard reaction i.e. HMF and furosine.

4.14.1 Effect of fortification on Maillard browning in milk (HMF content)

Most important type of browning in milk products results from the complex reactions following the interaction of carbonyl and amino compounds called Maillard browning. 5-hydroxymethylfurfural (HMF) was determined quantitatively by spectrophotometric measurement of 2-thiobarbituric acid reaction product (Keeney and Bassette 1958). HMF is potentially a polymer building block and is an indicator of the Maillard reaction (Lee and Nagy 1988). In fresh foods, the HMF level is close to zero (Babsky *et al*. 1986). However, it is found to be at a significant level in processed foods and hence is often used as a quality indicator (Lee and Nagy 1988). HMF is a well known decomposition product of 3-deoxyglucosones and has often served as an indicator of heat intensity applied to milk.

Different heat treatments were given to control and fortified milk samples viz. pasteurisation (63ºC/30 minutes), boiling and sterilisation (121ºC/15 minutes/15 psi) and their total HMF content was then determined. The HMF content of control and vitamin A+iron fortified milk samples are represented in the table 4.76. There was significant difference ($P<0.05$) in HMF content of raw, pasteurised, boiled and sterilised milk for each type of milk sample. There was non significant difference ($P<0.05$) in HMF content of raw control and fortified milk samples. Similarly, after pasteurisation, HMF content showed non significant difference ($P<0.05$) for all the milk samples except VA2500+FG25 fortified milk which differed significantly ($P<0.05$) from control milk but not from other fortified milk samples (HMF content (µM) was 2.853, 2.879, 2.895, 2.886 and 2.922 for control, VA2500+FPP20, VA2500+FPP25, VA2500+FG20 and VA2500+FG25 fortified milk samples, respectively). During boiling, HMF content of control, VA2500+FPP20 and

VA2500+FG20 fortified milk did not differ significantly ($P<0.05$) from each other, however, VA2500+FPP25 and VA2500+FG25 fortified milk samples differed significantly in terms of HMF content from control and FPP20 fortified milk, but not from each other and VA2500+FG20 fortified milk (2.924, 2.945, 2.981, 2.967 and 3.008 for control, VA2500+FPP20, VA2500+FPP25, VA2500+FG20 and VA2500+FG25 fortified milk samples, respectively). After sterilisation, all the samples differed significantly ($P<0.05$) from each other in terms of HMF content (10.585, 11.759, 14.574, 13.927, 16.527 for control, VA2500+FPP20, VA2500+FPP25, VA2500+FG20 and VA2500+FG25 fortified milk samples, respectively). In general, HMF content increased in milk after addition of vitamin A and iron salts and with increase in iron concentration. Moreover, HMF content of FG hydrate fortified milk was higher than that in FPP soluble fortified milk.

Table 4.76: Effect of iron fortification on total HMF content of milk

Heat treatment→ Sample↓	**HMF content in milk (μM)**			
	Raw milk	**Pasteurised milk**	**Boiled milk**	**Sterilised milk**
Control	2.312 ± 0.02^{aA}	2.853 ± 0.04^{bA}	2.924 ± 0.03^{cA}	10.585 ± 0.15^{dA}
VA2500+FPP20	2.312 ± 0.02^{aA}	2.879 ± 0.05^{bAB}	2.945 ± 0.03^{cA}	11.759 ± 0.04^{dB}
VA2500+FPP25	2.309 ± 0.01^{aA}	2.895 ± 0.04^{bAB}	2.981 ± 0.02^{cB}	14.574 ± 0.12^{dD}
VA2500+FG20	2.313 ± 0.02^{aA}	2.886 ± 0.05^{bAB}	2.967 ± 0.02^{cAB}	13.927 ± 0.02^{dC}
VA2500+FG25	2.311 ± 0.02^{aA}	2.922 ± 0.04^{bB}	3.008 ± 0.02^{cB}	16.527 ± 0.10^{dE}

Data are presented as means±SEM (n=3).

$^{a\text{-}b}$Means within rows with different lowercase superscript are significantly different ($P<0.05$) from each other.

$^{A\text{-}B}$Means within columns with different uppercase superscript are significantly different ($P<0.05$) from each other.

4.14.2 Effect of fortification on Maillard browning in milk (furosine content)

Different heat treatments were given to control and fortified milk samples viz. pasteurisation (63ºC/30 minutes), boiling and sterilisation (121ºC/15 minutes/15 psi) and their total furosine content was then determined. There was significant difference ($P<0.05$) in furosine content of raw, pasteurised, boiled and sterilised milk for each type of milk sample (table 4.77). There was non significant difference ($P<0.05$) in furosine content of raw control and fortified milk samples.

In pasteurised milk, control and VA2500+FPP20 fortified milk did not show any significant difference in furosine content, however, other fortified milk samples i.e. VA2500+FPP25, VA2500+FG20 and VA2500+FG25 differed significantly ($P<0.05$) from control and VA2500+FPP20 fortified milk in terms of furosine content, but did not differ from each other (8.687, 8.849, 9.068, 9.098 and 9.284 mg furosine/g protein for control, VA2500+FPP20, VA2500+FPP25, VA2500+FG20 and VA2500+FG25 fortified milk samples, respectively).

During boiling, control and VA2500+FPP20 fortified milk did not show any significant difference ($P<0.05$) in furosine content, however, other fortified milk samples i.e VA2500+FPP25, VA2500+FG20 and VA2500+FG25 differed significantly from control, VA2500+FPP20 fortified milk and amongst each other in terms of furosine content (9.527, 9.766, 10.065, 12.090 and 13.398 mg furosine/g protein for control, VA2500+FPP20, VA2500+FPP25, VA2500+FG20 and VA2500+FG25 fortified milk samples, respectively).

After sterilisation, all the samples except VA2500+FPP25 and VA2500+FG20 fortified milk differed significantly from each other in terms of furosine content (26.584, 30.581, 33.363, 33.494 and 35.983 for control, VA2500+FPP20, VA2500+FPP25, VA2500+FG20 and VA2500+FG25 fortified milk samples, respectively). In general, furosine content increased in milk after the addition of iron salts and with increase in their concentration, moreover, furosine content of FG hydrate fortified milk was higher than that in FPP soluble fortified milk.

Our findings were related to the findings of Birlouez-Aragon *et al*. (1997) who reported that the furosine content of growth milks (UHT milks supplemented with iron, vitamin C, lactose and linoleic acid to specify the nutritional needs of children) was 2-4 times higher than in UHT milks. Maillard reaction is clearly activated in growth milks, and is in agreement with the addition of lactose and iron to those milks.

Table 4.77: Effect of iron fortification on furosine content of milk

Heat treatment→ Sample↓	Furosine content in milk (mg/g protein)			
	Raw milk	**Pasteurised milk**	**Boiled milk**	**Sterilised milk**
Control	2.716±0.10^{aA}	8.687±0.26^{bA}	9.527±0.21^{cA}	26.584±0.69^{dA}
VA2500+FPP20	2.717±0.10^{aA}	8.849±0.26^{bA}	9.766±0.20^{cA}	30.581±0.78^{dB}
VA2500+FPP25	2.716±0.10^{aA}	9.068±0.27^{bB}	10.065±0.16^{cB}	33.363±0.50^{dC}
VA2500+FG20	2.717±0.10^{aA}	9.098±0.30^{bB}	12.090±0.52^{cC}	33.494±0.55^{dC}
VA2500+FG25	2.715±0.10^{aA}	9.284±0.29^{bB}	13.398±0.56^{cD}	35.983±0.26^{dD}

Data are presented as means±SEM (n=3).

$^{a-b}$Means within rows with different lowercase superscript are significantly different (P<0.05) from each other.

$^{A-B}$Means within columns with different uppercase superscript are significantly different (P<0.05) from each other.

4.15 Effect of fat content on quality characteristics of fortified milk

Whole milk, toned milk, double toned milk and skim milk were fortified with vitamin A acetate@2500 IU/L+FPP soluble@25 ppm (VA2500+FPP25) and vitamin A acetate@2500 IU/L+FG hydrate@25 ppm (VA2500+FG25) and effect of fat content and fortification with vitamin A and iron on sensory acceptability, viscosity, curd tension and oxidative stability was then evaluated.

4.15.1 Effect of fat on sensory acceptability of milk

It has been shown that perceptions such as perceived sensory properties, cost, convenience, habit, nutritional knowledge and beliefs and concerns about fat and cholesterol contents influence milk consumption and attitudes towards the different types of milk. Taste is a major determinant of food choice (Bus and Worsley 2002).

4.15.1.1 Sensory acceptability of whole milk

Whole milk was fortified with vitamin A acetate@2500 IU/L+FPP soluble@25 ppm (VA2500+FPP25) and vitamin A acetate@2500 IU/L+FG hydrate@25 ppm (VA2500+FG25) and was evaluated for sensory acceptability by a panel of 10 judges. The overall sensory scores obtained for VA2500+FPP25 and VA2500+FG25 fortified milk samples were 94.95 and 94.80, respectively as compared to 95.14 obtained for control milk (table 4.78). Statistically, there was non significant difference ($P<0.05$) in colour and appearance, odour, taste, mouthfeel and total scores of control and fortified milk samples.

Table 4.78: Sensory acceptability of whole milk

Characteristics	Maximum score	Sample		
		Control	VA2500+FPP25	VA2500+FG25
Colour and appearance	10	9.60 ± 0.04^{a}	9.57 ± 0.03^{a}	9.57 ± 0.03^{a}
Odour	20	18.80 ± 0.10^{a}	18.78 ± 0.09^{a}	18.72 ± 0.10^{a}
Taste	40	38.15 ± 0.11^{a}	38.06 ± 0.11^{a}	38.01 ± 0.10^{a}
Mouthfeel	30	28.60 ± 0.14^{a}	28.54 ± 0.14^{a}	28.49 ± 0.15^{a}
Total score	100	95.14 ± 0.21^{a}	94.95 ± 0.21^{a}	94.8 ± 0.22^{a}

Data are presented as means±SEM (n=30).
$^{a-b}$Means with same superscript in a row do not vary significantly ($P<0.05$) from each other.

4.15.1.2 Sensory acceptability of toned milk

Toned milk was fortified with vitamin A acetate@2500 IU/L+FPP soluble@25 ppm (VA2500+FPP25) and vitamin A acetate@2500 IU/L+FG hydrate@25 ppm (VA2500+FG25) and was evaluated for sensory acceptability by a panel of 10 judges. The overall sensory scores obtained for VA2500+FPP25 and VA2500+FG25 fortified milk samples were 94.59 and 94.47, respectively as compared to obtained 94.76 for control milk (Table 4.79). Statistically, there was non significant difference ($P<0.05$) in colour and appearance, odour, taste, mouthfeel and total scores of control and fortified milk samples.

Table 4.79: Sensory acceptability of toned milk

Characteristics	Maximum score	Sample		
		Control	VA2500+FPP25	VA2500+FG25
Colour and appearance	10	9.26 ± 0.10^{a}	9.21 ± 0.10^{a}	9.17 ± 0.10^{a}
Odour	20	18.71 ± 0.07^{a}	18.65 ± 0.07^{a}	18.61 ± 0.07^{a}
Taste	40	38.54 ± 0.09^{a}	38.44 ± 0.09^{a}	38.44 ± 0.10^{a}
Mouthfeel	30	28.25 ± 0.12^{a}	28.29 ± 0.10^{a}	28.25 ± 0.10^{a}
Total score	100	94.76 ± 0.19^{a}	94.59 ± 0.17^{a}	94.47 ± 0.18^{a}

Data are presented as means±SEM (n=30).
$^{a-b}$Means with same superscript in a row do not vary significantly ($P<0.05$) from each other.

4.15.1.3 Sensory acceptability of double toned milk

Double toned milk was fortified with vitamin A acetate@2500 IU/L+FPP soluble@25 ppm (VA2500+FPP25) and vitamin A acetate@2500 IU/L+FG hydrate@25 ppm (VA2500+FG25) and was evaluated for sensory acceptability by a panel of 10 judges. The overall sensory scores obtained for VA2500+FPP25 and VA2500+FG25 fortified milk samples were 91.56 and 91.31, respectively as compared to 91.79 obtained for control milk (Table 4.80). Statistically, there was non significant difference ($P<0.05$) in colour and appearance, odour, taste, mouthfeel and total scores of control and fortified milk samples.

Table 4.80: Sensory acceptability of double toned milk

Characteristics	Maximum score	Sample		
		Control	VA2500+FPP25	VA2500+FG25
Colour and appearance	10	8.93±0.1^{a}	8.89±0.09^{a}	8.86±0.09^{a}
Odour	20	18.17±0.09^{a}	18.11±0.08^{a}	18.04±0.09^{a}
Taste	40	37.01±0.11^{a}	36.95±0.11^{a}	36.89±0.11^{a}
Mouthfeel	30	27.68±0.12^{a}	27.62±0.12^{a}	27.53±0.13^{a}
Total score	100	91.79±0.25^{a}	91.56±0.24^{a}	91.31±0.25^{a}

Data are presented as means±SEM (n=30).
$^{a-b}$Means with same superscript in a row do not vary significantly ($P<0.05$) from each other.

4.15.1.4 Sensory acceptability of skim milk

Skim milk was fortified with vitamin A acetate@2500 IU/L+FPP soluble@25 ppm (VA2500+FPP25) and vitamin A acetate@2500 IU/L+FG hydrate@25 ppm (VA2500+FG25) and was evaluated for sensory acceptability by a panel of 10 judges. The overall sensory scores obtained for VA2500+FPP25 and VA2500+FG25 fortified milk samples were 87.32 and 86.29, respectively as compared to 88.00 obtained for control milk (Table 4.81). Statistically, control milk and VA2500+FPP25 fortified skim milk showed non significant difference in terms of all sensory attributes studied ($P<0.05$). VA2500+FG25 milk differed from control milk in terms of taste, mouthfeel and total scores, however, there was non significant difference among all other parameters (colour and appearance and odour).

Table 4.81: Sensory acceptability of skim milk

Characteristics	Maximum score	Sample		
		Control	VA2500+FPP25	VA2500+FG25
Colour and appearance	10	8.43±0.09^{a}	8.39±0.09^{a}	8.32±0.10^{a}
Odour	20	18.06±0.07^{a}	17.99±0.07^{a}	17.93±0.07^{a}
Taste	40	35.40±0.13^{a}	35.12±0.12^{a}	34.78±0.13^{b}
Mouthfeel	30	26.11±0.40^{a}	25.82±0.39^{ab}	25.26±0.41^{b}
Total score	100	88.00±0.45^{a}	87.32±0.44^{a}	86.29±0.46^{b}

Data are presented as means±SEM (n=30).
$^{a\text{-}b}$Means with same superscript in a row do not vary significantly (P<0.05) from each other.

Our results were in consonance with Brewer *et al*. (1999) who reported that the subjects preferred whole milk to skimmed milk, and the sensory score for liking increased with increasing fat content. Ricahrdson-Harman *et al*. (2000) also found a positive relationship between creaminess and preference, with products higher in fat being perceived as creamiest. Wham (2000) reported that the majority of respondents had positive attitudes towards the taste of milk, but no distinctions between types of milk were made. Whole milk received highest agreement on taste and mouthfeel perceptions, although reduced fat milk closely followed (Bus and Worsley 2002). The relatively small differences in sensory evaluation between reduced fat milk and whole milk might be explained by the fact that fat does not affect the sensory properties in a linear manner (Frost *et al*. 2000).

4.15.2 Effect of fat on viscosity of control and fortified milk

Viscosity is the resistance to flow. Several factors influence viscosity including the temperature, concentrations and state of the casein micelles and fat globules. Viscosity affects the organoleptic properties like mouthfeel and flavour (Chandan and Kilara 2011). There was significant difference (P<0.05) in viscosity of whole milk, toned milk, double toned milk and skim milk for control and fortified samples (table 4.82). However, there was non significant difference (P<0.05) in viscosity of control and fortified samples for all types of milks i.e. whole milk, toned milk, double toned milk and skim milk.

The viscosity and rheological behaviour of milk largely depend on the state and concentration of fat. Janness and Patton (1959) reported that representative values of viscosity at 20ºC of whole milk and skim milk were 2.127 and 1.790 mPa.s, respectively i.e. viscosity of whole milk was higher than skim milk at a constant temperature. There are no reports available regarding the impact of addition of iron salts on viscosity of milk. However, Gaucheron *et al.* (1997a) reported that there was protein coagulation at 4.0 mM concentration iron, which could possibly affect the viscosity of fortified milk. Dhaka (1982) observed that salts which disperse the casein micelles increase viscosity of milk. Change in the viscosity of milk was not due to change in pH, it was due to the specific property of ions of salts.

Table 4.82: Effect of fat on viscosity (cP) of control and fortified milk

Milk type→ Sample type↓	**Whole milk**	**Toned milk**	**Double toned milk**	**Skim milk**
Control	2.01 ± 0.08^{aA}	1.91 ± 0.08^{bA}	1.82 ± 0.08^{cA}	1.75 ± 0.07^{dA}
VA2500+FPP25	2.01 ± 0.08^{aA}	1.91 ± 0.08^{bA}	1.82 ± 0.07^{cA}	1.75 ± 0.07^{dA}
VA2500+FG25	2.02 ± 0.08^{aA}	1.91 ± 0.08^{bA}	1.83 ± 0.08^{cA}	1.76 ± 0.07^{dA}

Data are presented as means±SEM (n=3).

$^{a-b}$Means within rows with different lowercase superscript are significantly different ($P<0.05$) from each other.

$^{A-B}$Means within columns with different uppercase superscript are significantly different ($P<0.05$) from each other.

4.15.3 Effect of fat on curd tension of control and fortified milk

There was significant difference ($P<0.05$) in curd tension of whole milk, toned milk, double toned milk and skim milk for control and fortified samples. However, non significant difference ($P<0.05$) was observed in curd tension of control and fortified samples for all types of milks i.e. whole milk, toned milk, double toned milk and skim milk (table 4.83). Chandrasekhara *et al*. (1957) reported that skimming brought a slight increase in curd tension values of milk samples. There are no reports regarding effect of iron on curd tension of milk.

However, our findings were related to findings of Gaucheron *et al.* (1997a) on curd firmness. They had reported decrease in curd firmness of skim milk fortified with $FeCl_2$ and slight decrease in case of $FeCl_3$ fortification and suggested that the secondary (aggregation of paracasein micelles) and tertiary (gel formation) phases could be altered by modifications of molecular interactions between paracasein micelles in the presence of iron ions which might have led to slight decrease in curd firmness.

Table 4.83: Effect of fat on curd tension (g) of control and fortified milk

Milk type→ Sample type↓	**Whole milk**	**Toned milk**	**Double toned milk**	**Skim milk**
Control	35.7434 ±0.20^{aA}	37.2963 ±0.15^{bA}	38.6476 ±0.16^{cA}	40.6638 ±0.23^{dA}
VA2500+FPP25	35.7479 ±0.20^{aA}	37.3038 ±0.15^{bA}	38.6577 ±0.16^{cA}	40.6759 ±0.23^{dA}
VA2500+FG25	35.7485 ±0.20^{aA}	37.3045 ±0.15^{bA}	38.6583 ±0.16^{cA}	40.6842 ±0.24^{dA}

Data are presented as means±SEM (n=3).
$^{a-b}$Means within rows with different lowercase superscript are significantly different ($P<0.05$) from each other.
$^{A-B}$Means within columns with different uppercase superscript are significantly different ($P<0.05$) from each other.

4.15.4 Effect of fat on oxidative stability of control and fortified milk (TBA value)

Dairy products are known to oxidise during processing and storage, resulting in deterioration of flavour and nutritional value (Hedegaard *et al*. 2006). Effect of fortification and fat content was evaluated on oxidative stability of milk in terms of TBA value. There was significant difference ($P<0.05$) in TBA value of whole milk, toned milk, double toned milk and skim milk for control and fortified samples (table 4.84).

Similarly, the control and fortified milk whole and toned milk samples differed significantly ($P<0.05$) from each other in terms of TBA value (TBA value of whole milk was 0.021, 0.025 and 0.026 for control, VA2500+FPP25 and VA2500+FG25 fortified milk, respectively; for toned milk was 0.019, 0.021 and 0.023 for control, VA2500+FPP25 and VA2500+FG25 fortified milk, respectively). Control and VA2500+FPP25 fortified double toned milk showed non significant difference ($P<0.05$) in TBA value (0.010 and 0.010, respectively), however these samples differed significantly from VA2500+FG25 fortified double toned milk (0.011). There was non significant difference ($P<0.05$) amongst control, VA2500+FPP25 and VA2500+FG25 fortified skim milk (0.006) for all samples.

Wang and King (1973) reported that there was an increase in TBA value of milk when iron salts were added. Kehagias and Radhma (1973) stated that under certain circumstances, particularly in products with a low protein but high milk fat content, the presence of iron causes oxidative deterioration. However, Ravikiran *et al.* (1977) reported that increasing ferric lactose concentration upto 30 ppm concentration did not increase TBA value of milk.

Table 4.84: Effect of fat on oxidative stability of control and fortified milk (TBA value)

Milk type→ Sample type↓	Whole milk	Toned milk	Double toned milk	Skim milk
Control	0.021 ± 0.0008^{aA}	0.019 ± 0.0008^{bA}	0.010 ± 0.0006^{cA}	0.006 ± 0.0003^{dA}
VA2500+FPP25	0.025 ± 0.0012^{aB}	0.021 ± 0.0013^{bB}	0.010 ± 0.0007^{cA}	0.006 ± 0.0003^{dA}
VA2500+FG25	0.026 ± 0.0008^{aC}	0.023 ± 0.0015^{bC}	0.011 ± 0.0007^{cB}	0.006 ± 0.0003^{dA}

Data are presented as means±SEM (n=3).

$^{a\text{-}b}$Means within rows with different lowercase superscript are significantly different ($P<0.05$) from each other.

$^{A\text{-}B}$Means within columns with different uppercase superscript are significantly different ($P<0.05$) from each other.

4.16 Effect of fortification on sensory, physical and microbiological properties of *dahi*

4.16.1 Sensory analysis scores of *dahi* made from control and vitamin A+iron fortified milk

4.16.1.1 Sensory analysis scores of *dahi* made from control and VA+FPP fortified milk

Dahi samples were prepared from control milk and fortified milk (vitamin A acetate@2500 IU/L+FPP soluble@15, 20 and 25 ppm iron) and were given to a panel of 10 judges for sensory analysis. All *dahi* samples were comparable to each other and control *dahi* in all sensory aspects and their overall scores were also comparable to control (93.10, 92.71, 92.43 and 92.15 for control, VA2500+FPP15, VA2500+FPP20 and VA2500+FPP25 fortified *dahi* samples) as shown in table 4.85. Statistically also, the differences in the sensory attributes studied for all *dahi* samples were non significant ($P<0.05$).

Table 4.85: Sensory analysis scores of *dahi* made from control and VA@2500 IU/L+FPP soluble@15, 20 and 25 ppm fortified milk

Characteristics	Maximum score	VA2500+FPP			
		Iron concentration (ppm)			
		Control	15	20	25
Flavour	45	41.86±0.34^a	41.67±0.34^a	41.49±0.36^a	41.33±0.36^a
Body and texture	30	28.34±0.12^a	28.21±0.12^a	28.19±0.12^a	28.14±0.12^a
Acidity	10	9.19±0.06^a	9.15±0.06^a	9.11±0.06^a	9.07±0.07^a
Colour and appearance	10	8.97±0.07^a	8.93±0.07^a	8.91±0.07^a	8.87±0.07^a
Container and closure	05	4.74±0.02^a	4.74±0.02^a	4.74±0.02^a	4.74±0.02^a
Total score	100	93.10±0.48^a	92.71±0.48^a	92.43±0.49^a	92.15±0.48^a

Data are presented as means±SEM (n=30).

$^{a-b}$Means with same superscript in a row do not vary significantly (P<0.05) from each other.

4.16.1.2 Sensory analysis scores of *dahi* made from control and VA+FG fortified milk

Dahi samples were prepared from control milk and fortified milk (VA2500 IU/L+FG@15, 20 and 25 ppm) and were given to a panel of 10 judges for sensory analysis. All *dahi* samples were comparable to each other and control in the sensory aspects and their overall scores were also comparable to control (94.48, 94.27, 93.97 and 93.83 for control, VA2500+FG15, VA2500+FG20 and VA2500+FG25 fortified *dahi* samples) as shown in table 4.86. Statistically also, the differences in all sensory attributes and total scores of all *dahi* samples (control as well as fortified) were non significant (P<0.05).

Yoghurt is the Western counterpart of *dahi*. No reports are available in literature related to studies on *dahi* prepared from iron fortified milk. However, results can be correlated with the similar types of studies conducted for yoghurt.

Our results were in accordance with El-Behairy and Mohamed (2010) who reported that yoghurt samples were acceptable upto 20 and 40 mg/kg ferric chloride and 20 mg/kg ferrous sulphate. Simova *et al.* (2008) also reported that iron-fortified yoghurts (ferrous lactate) did not differ significantly in their characteristics from unfortified yoghurt. Hekmat and McMahon (1997) reported that yoghurts (non fat and low fat) made from iron fortified milk (casein chelated iron and whey protein-chelated iron@20 ppm) had slightly higher oxidised flavour scores than did the control yoghurt as detected by trained panellists. There was no increase in metallic, bitter, or other off-flavours. The consumer panel did not detect any significant differences in the appearance, mouthfeel, flavour or overall quality between fortified and unfortified flavoured yoghurts. All yoghurt samples were liked by the consumer panellists. They suggested that yoghurt is a suitable vehicle for iron fortification.

Table 4.86: Sensory analysis scores of *dahi* made from control and VA@2500 IU/L+FG hydrate@15, 20 and 25 ppm fortified milk

Characteristics	Maximum Score	VA2500+FG			
		Iron concentration (ppm)			
		Control	15	20	25
Flavour	45	43.35±0.26^{a}	43.24±0.26^{a}	43.1±0.26^{a}	43.06±0.25^{a}
Body and texture	30	28.57±0.10^{a}	28.52±0.10^{a}	28.45±0.10^{a}	28.41±0.11^{a}
Acidity	10	8.85±0.09^{a}	8.85±0.10^{a}	8.79±0.10^{a}	8.72±0.10^{a}
Colour and appearance	10	8.99±0.09^{a}	8.95±0.09^{a}	8.91±0.09^{a}	8.91±0.09^{a}
Container and closure	05	4.72±0.03^{a}	4.72±0.03^{a}	4.72±0.03^{a}	4.72±0.03^{a}
Total score	100	94.48±0.34^{a}	94.27±0.33^{a}	93.97±0.33^{a}	93.83±0.32^{a}

Data are presented as means±SEM (n=30).
$^{a-b}$Means with same superscript in a row do not vary significantly ($P<0.05$) from each other.

Umbelino *et al*. (2001) also showed that the addition of iron (microencapsulated ferrous sulphate) do not interfere in the sensory properties of soy yoghurt. Achanta *et al*. (2007) reported that non significant ($P>0.05$) differences was observed for flavour and appearance scores of the yoghurts fortified with the iron (ferric orthophosphate) compared to the control. Umbelino and Rossi (2009) reported that the addition of iron (microencapsulated ferrous sulphate@12 ppm iron) did not significantly affect the sensory scores for all evaluated attributes. Azzam (2009) reported that the source of iron used for fortification had non significant effect on smoothness of yoghurt samples when fresh and during storage.

It is well known that major off-flavours may be associated with fortified dairy milk products: oxidised flavour resulting from catalysis of lipid oxidation by iron and metallic flavour contributed by iron salts (Jackson and Lee 1992). No oxidative rancidity was detected in fresh yoghurt and during storage samples (Abd-Rabou *et al*. 1999; Osman and Ismail 2004) while, Mehanna *et al*. (2000) concluded that fresh yoghurt fortified with iron from different sources seem to be slightly affected by iron concentration.

El-Kholy *et al.* (2011) reported that the sensory quality of iron-fortified dairy foods has been shown to be affected by the type of iron salt used. They showed that the highest flavour score was for control and yoghurt fortified with iron-whey protein chelate (Fe-WP) when fresh and during storage. The lowest score was for yoghurt fortified with ammonium ferrous sulphate which showed a slight metallic flavour. Also, the iron treatments did not affect significantly the body and texture of yoghurt. The colour and appearance of yoghurt fortified with iron was not affected by the sources of iron used (ammonium ferric sulphate, ammonium ferrous sulphate, iron-casein complex and iron-whey protein complex). The total score of yoghurt fortified with iron from different sources showed that the addition of Fe-WP resulted in yoghurt quite similar to that of control yoghurt.

4.16.2 Effect of milk fortification on pH, acidity, water holding capacity (WHC) and syneresis of *dahi*

Appropriate acid concentration is one the important factors ensuring quality of fermented milk. There was a slight but statistically non significant difference ($P<0.05$) in pH of *dahi* samples made from control and fortified milk, pH of fortified ones being slightly lower than the control samples (table 4.87). Control, VA2500+FPP15 and VA2500+FPP20 *dahi* samples did not differ significantly ($P<0.05$) in terms of acidity, however, VA2500+FPP25, VA2500+FG15, VA2500+FG20 and VA2500+FG25 samples were significantly different from control *dahi*, though the difference was not very high. There was non significant difference ($P<0.05$) in acidity values of VA2500+FPP15, VA2500+FPP20, VA2500+FPP25 and VA2500+FG15 *dahi* samples; and VA2500+FPP25, VA2500+FG15, VA2500+FG20 and VA2500+FG25 *dahi* samples also did not significantly differ from each other (table 4.87). All *dahi* samples showed non significant difference ($P<0.05$) from each other in terms of water holding capacity (table 4.87). Further, fortified *dahi* samples showed non significant difference ($P<0.05$) in syneresis values from control *dahi* except VA2500+FG25 *dahi*, however, there was non significant difference between syneresis values of VA2500+FG25 *dahi* and VA2500+FPP20, VA2500+FPP25, VA2500+FG15, VA2500+FG20 *dahi* samples (table 4.87).

No reports are available in literature related to study of *dahi* prepared from iron fortified milk. However, results can be correlated to the similar types of studies conducted for yoghurt. Our results are in accordance with El-Behairy and Mohamed (2010) who reported a decrease in pH and increase in acidity of yoghurt samples prepared from iron fortified milk compared to those prepared from control milk was observed. El-Kholy *et al.* (2011) reported that the changes in the pH values for all yoghurt treatments with iron (Fe-WP complex) were not significantly ($p<0.05$) different.

However, Umbelino and Rossi (2009) reported that the initial titratable acidity of control soy yoghurt (0.87±0.01% lactic acid) was higher than of fortified product (0.85±0.01% lactic acid). They also showed that unfortified (control) and iron fortified soy yoghurt required 4.5 and 5.15 hrs to reduce pH to the 4.4-4.5 range, respectively, indicating that the starter culture was capable to produce enough acids in a suitable time period and that the fortification do not make the fermentation process unviable.

Our findings were in accordance with Achanta *et al.* (2007) who studied the rheological properties of yoghurts fortified with various minerals and reported that the mean syneresis values of yoghurts fortified with iron (ferric orthophosphate) were significantly ($P<0.05$) lower than in the control yoghurt. They reported that yoghurt fortified with iron had better water holding capacities compared to the control.

Gaucheron *et al.* (1997b) reported that there may be a change in casein structure when iron binds to micellar casein. This probable change in the structure of casein may explain better water holding capacity/less released whey in iron fortified yoghurts. El-Kholy *et al.* (2011) showed that there was non significant difference in syneresis among most of yoghurt fortified with different iron salts viz. ammonium ferric sulphate, ammonium ferrous sulphate, iron-casein complex and iron-whey protein complex.

Table 4.87: Effect of milk fortification on pH, acidity, WHC and syneresis of *dahi*

Sample	pH	Acidity (%)	WHC (%)	Syneresis (%)
Control	4.53±0.03^{a}	0.772±0.007^{a}	77.94±0.41^{a}	3.93±0.09^{a}
VA2500+FPP15	4.52±0.03^{a}	0.779±0.006^{ab}	78.09±0.34^{a}	3.83±0.15^{a}
VA2500+FPP20	4.51±0.03^{a}	0.779±0.009^{ab}	78.12±0.35^{a}	3.80±0.12^{ab}
VA2500+FPP25	4.50±0.04^{a}	0.786±0.005^{bc}	78.20±0.38^{a}	3.63±0.19^{ab}
VA2500+FG15	4.51±0.03^{a}	0.784±0.004^{bc}	78.06±0.36^{a}	3.77±0.15^{ab}
VA2500+FG20	4.50±0.04^{a}	0.787±0.003^{bc}	78.16±0.34^{a}	3.67±0.12^{ab}
VA2500+FG25	4.48±0.03^{a}	0.792±0.004^{c}	78.30±0.37^{a}	3.50±0.20^{b}

Data are presented as means±SEM (n=3).
$^{a-b}$Means with same superscript in a column do not vary significantly (P<0.05) from each other.

4.16.3 Effect of milk fortification on diacetyl content, setting time and Lactococcal count of *dahi*

Gandhi and Natrajan (2010) reported that diacetyl is main flavour component in *dahi*. Diacetyl content of *dahi* was determined to evaluate the effect of iron fortification on flavour producing activity of *dahi* cultures. There was a slight but statistically non significant difference (P<0.05) in diacetyl content of *dahi* samples made from control and fortified milk, being slightly higher in fortified sample than the control samples. All *dahi* samples showed non significant difference (P<0.05) in setting time and Lactococcal count (table 4.88). However, the results indicated that setting time for *dahi* decreased slightly though statistically non significantly (P<0.05) on addition of iron salts, and setting time being lesser for FG hydrate fortified *dahi* than FPP soluble. On the other hand, Lactococcal count of prepared from iron fortified milk was slightly more than that from control milk.

No reports are available in literature related to study of *dahi* prepared from iron fortified milk. However, results were correlated to the similar types of studies conducted for yoghurt. El-Kholy *et al.* (2011) reported that diacetyl content of yoghurt increased after addition of iron (Fe-WP) to milk and the effect was statistically significant. Our results are in accordance with El-Behairy and Mohamed (2010) who reported higher lactic acid count in yoghurt prepared from iron fortified milk (ferric chloride and ferrous sulphate). However, Simova *et al.* (2008) reported that iron fortification of milk with ferrous lactate @8, 15 and 27 ppm iron had no effect on the incubation time required for the yoghurt mixes to reach pH 4.5±0.1 and it did not affect significantly the growth and viability of *L. delbruekii* subsp. *bulgaricus* and *S. thermophilus* during milk fermentation and storage of yoghurt. Lactic acid bacteria are reported to be exception among living organisms in that they show no iron requirements (Bruyneel *et al*. 1989; Cervaux *et al*. 2000; Weinberg 1997). There are some reports that some *Lactobacillus* species have the ability to bind ferric hydroxide at their cell surface, rendering it unavailable to pathogenic microorganisms.

Hekmat and McMahon (1997) also reported that counts of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* after 1 day of storage in iron-fortified skim yoghurts (casein chelated iron and whey protein-chelated iron@20 ppm) were not significantly different from numbers in unfortified yoghurts. Iron fortification had no effect on the incubation time required for the yoghurt mixes to reach pH 4.3. El-Kholy *et al*. (2011) showed that fortification of yoghurt with different iron salts had no effect on the total lactic acid bacteria in all treatments. Ocak and Köse (2010) found that iron fortification with ferric nitrate (@0.3 and 0.6 ppm) had no effect on the incubation time required for the yoghurt mixes to reach pH 4.5 ± 0.1.

Table 4.88: Effect of milk fortification on diacetyl content, setting time and Lactococcal count of *dahi*

Sample	Diacetyl (mg/g)	Setting time (hrs)	Lactococcal count (10^7 cfu/ml)
Control	3.169±0.049^a	8.07±0.15^a	140.3±7.535^a
VA2500+FPP15	3.171±0.048^a	8.03±0.15^a	146.0±5.29^a
VA2500+FPP20	3.174±0.048^a	7.97±0.15^a	148.3±4.67^a
VA2500+FPP25	3.182±0.047^a	7.87±0.15^a	151.7±4.06^a
VA2500+FG15	3.170±0.049^a	8.00±0.17^a	145.7±6.17^a
VA2500+FG20	3.174±0.048^a	7.97±0.15^a	148.3±6.33^a
VA2500+FG25	3.181±0.046^a	7.83±0.15^a	150.3±5.84^a

Data are presented as means±SEM (n=3).
$^{a-b}$Means with same superscript in a column do not vary significantly (P<0.05) from each other.

4.16.4 Effect of milk fortification on viscosity of *dahi*

Viscosity of *dahi* prepared from fortified milk was slightly more than control *dahi* (table 4.89). At 10, 20 and 30 rpm, viscosity of all the samples showed statistically non significant difference (P<0.05) from each other.

Our results were in accordance with Achanta *et al*. (2007) who reported that there was a slight but non significant increase in viscosity of yoghurt samples fortified with iron (ferric orthophosphate). Umbelino *et al*. (2001) reported that addition of iron did not alter the viscosity of soy yoghurt. Umbelino and Rossi (2009) reported that storage did not have any significant effect on viscosity and consistency of the soy yoghurt fortified or not with iron (microencapsulated ferrous sulphate). The average consistency did not vary between the samples.

However, viscosity of iron soy yoghurt was lower than control soy yoghurt (p<0.05), during all the storage period. Their results revealed that, despite the differences in rheological properties, the control and iron fortified soy yoghurt presented a stable gel with characteristic of natural set yoghurt. Rice and McMahon (1998) reported that there was no effect of iron on the apparent viscosity of mozzarella cheese fortified with iron at 25 mg/kg.

Table 4.89: Effect of milk fortification on viscosity of *dahi*

Sample	Viscosity at 10 rpm (cP)	Viscosity at 20 rpm (cP)	Viscosity at 30 rpm (cP)
Control	3305.47±96.49^a	1777.47±56.17^a	1267.70±42.01^a
VA2500+FPP15	3308.87±95.00^a	1792.27±52.50^a	1284.53±39.46^a
VA2500+FPP20	3324.93±93.90^a	1821.00±43.51^a	1296.17±38.14^a
VA2500+FPP25	3359.43±102.58^a	1855.47±51.57^a	1313.93±41.59^a
VA2500+FG15	3314.20±93.75^a	1807.77±49.78^a	1295.13±39.50^a
VA2500+FG20	3333.57±94.66^a	1834.57±42.17^a	1316.03±36.94^a
VA2500+FG25	3374.50±104.60^a	1867.60±54.64^a	1326.00±40.33^a

Data are presented as means±SEM (n=3).

$^{a-b}$Means with same superscript in a column do not vary significantly (P<0.05) from each other.

4.16.5 Effect of milk fortification on texture profile of *dahi*

Control and fortified *dahi* samples showed non significant difference (P<0.05) in firmness except VA2500+FG25 *dahi* sample (table 4.90), however, VA2500+FG25 sample did not differ significantly from VA2500+FPP20, VA2500+FPP25, VA2500+FG15 and VA2500+FG20. In terms of work of adhesion, VA2500+FG25 *dahi* sample differed significantly (P<0.05) from all other *dahi* samples except VA2500+FG20, however, all other *dahi* samples showed non significant difference from each other. There was no significance difference observed in work of shear for all *dahi* samples. Significant difference was observed in stickiness of control and fortified *dahi* sample ($P<0.05$).

Our results were in consonance with Ocak and Köse (2010) who reported that addition of iron (ferric nitrate@0.3 and 0.6 ppm) to milk caused a general increase in firmness and cohesiveness of yoghurt in comparison to the control yoghurt and this was found statistically significant ($P<0.01$). Sandoval-Castilla *et al.* (2004) stated that the protein matrix was responsible for the firmness and springiness of yoghurt.

Table 4.90: Effect of milk fortification on texture profile of *dahi*

Sample	Firmness N	Work of adhesion N.s	Work of shear N.s	Stickiness N
Control	1.275 ± 0.105^{a}	-1.427 ± 0.092^{a}	10.568 ± 0.562^{a}	-0.227 ± 0.014^{a}
VA2500+FPP15	1.262 ± 0.125^{a}	-1.402 ± 0.091^{a}	10.891 ± 0.426^{a}	-0.204 ± 0.010^{b}
VA2500+FPP20	1.371 ± 0.066^{ab}	-1.378 ± 0.089^{a}	11.018 ± 0.432^{a}	0.189 ± 0.006^{bc}
VA2500+FPP25	1.420 ± 0.060^{ab}	-1.317 ± 0.094^{a}	11.145 ± 0.416^{a}	-0.181 ± 0.007^{c}
VA2500+FG15	1.422 ± 0.093^{ab}	-1.288 ± 0.093^{a}	11.008 ± 0.434^{a}	-0.196 ± 0.010^{bc}
VA2500+FG20	1.483 ± 0.116^{ab}	-1.247 ± 0.092^{ab}	11.101 ± 0.423^{a}	-0.184 ± 0.007^{c}
VA2500+FG25	1.576 ± 0.126^{b}	-0.863 ± 0.394^{b}	11.424 ± 0.505^{a}	-0.170 ± 0.006^{c}

Data are presented as means±SEM (n=3).
$^{a-b}$Means with same superscript in a column do not vary significantly ($P<0.05$) from each other.

4.17 Effect of fortification on sensory, physical and microbiological properties of yoghurt

4.17.1 Sensory analysis scores of yoghurt made from control and vitamin A+iron fortified milk

4.17.1.1 Sensory analysis scores of yoghurt made from control and VA+FPP fortified milk

Yoghurt samples were prepared from control milk and fortified milk (vitamin A acetate@2500 IU/L+FPP soluble@15, 20 and 25 ppm) and were given to a panel of 10 judges for sensory analysis. All yoghurt samples were comparable to each other and control in all sensory aspects and their overall scores were also comparable to control (92.84, 92.60, 92.35 and 92.17 for control, VA2500+FPP15, VA2500+FPP20 and VA2500+FPP25 fortified yoghurt samples) as shown in table 4.91. Statistically also, the difference in total scores and scores of all yoghurt samples for other parameters was non significant ($P<0.05$).

Table 4.91: Sensory analysis scores of yoghurt made from control and VA@2500 IU/L+FPP soluble@15, 20 and 25 ppm fortified milk

Characteristics	Maximum score	VA2500+FPP			
		Iron concentration (ppm)			
		Control	15	20	25
Flavour	45	42.38±0.35^{a}	42.26±0.35^{a}	42.16±0.36^{a}	42.09±0.36^{a}
Body and texture	30	28.40±0.14^{a}	28.34±0.13^{a}	28.28±0.14^{a}	28.26±0.14^{a}
Acidity	10	8.72±0.07^{a}	8.72±0.07^{a}	8.68±0.07^{a}	8.64±0.07^{a}
Colour and appearance	10	8.69±0.09^{a}	8.64±0.09^{a}	8.58±0.09^{a}	8.54±0.09^{a}
Container and closure	05	4.65±0.03^{a}	4.65±0.03^{a}	4.65±0.03^{a}	4.65±0.03^{a}
Total score	100	92.84±0.43^{a}	92.60±0.42^{a}	92.35±0.43^{a}	92.17±0.43^{a}

Data are presented as means±SEM (n=30).
$^{a-b}$Means with same superscript in a row do not vary significantly (P<0.05) from each other.

4.17.1.2 Sensory analysis scores of yoghurt made from control and VA+FG fortified milk

Yoghurt samples were prepared from control milk and fortified milk (VA2500+FG@15, 20 and 25 ppm) and were given to a panel of 10 judges for sensory analysis. All yoghurt samples were comparable to each other and control in all sensory aspects and their overall scores were also comparable to control (93.37, 92.96, 92.61 and 92.55 for control, VA2500+FG15, VA2500+FG20 and VA2500+FG25 fortified yoghurt samples) as shown in table 4.92. Statistically also, the differences in total scores and scores of all yoghurt samples for other parameters were non significant (P<0.05).

Our results were in accordance with El-Behairy and Mohamed (2010) who reported that yoghurt samples were acceptable upto 20 and 40 mg/kg ferric chloride and 20 mg/kg ferrous sulphate. Simova *et al.* (2008) also reported that iron-fortified yoghurts (ferrous lactate) did not differ significantly in their characteristics from unfortified yoghurt. Hekmat and McMahon (1997) reported that yoghurts (non fat and low fat) made from iron fortified milk (casein chelated iron and whey protein-chelated iron@20 ppm) had slightly higher oxidised flavour scores than did the control yoghurt as detected by trained panellists. There was no increase in metallic, bitter, or other off-flavours. The consumer panel did not detect any significant differences in the appearance, mouthfeel, flavour or overall quality between fortified and unfortified flavoured yoghurts. All yoghurt samples were liked by the consumer panellists. They suggested that yoghurt is a suitable vehicle for iron fortification.

Table 4.92: Sensory analysis scores of yoghurt made from control and VA@2500 IU/L+FG hydrate@15, 20 and 25 ppm fortified milk

Characteristics	**Maximum score**	**VA2500+FG**			
		Iron concentration (ppm)			
		Control	**15**	**20**	**25**
Flavour	45	42.38±0.33^a	42.27±0.32^a	42.14±0.33^a	42.14±0.33^a
Body and texture	30	28.67±0.13^a	28.5±0.13^a	28.43±0.13^a	28.42±0.13^a
Acidity	10	9.02±0.10^a	8.96±0.10^a	8.88±0.10^a	8.86±0.10^a
Colour and appearance	10	8.63±0.10^a	8.56±0.10^a	8.48±0.10^a	8.46±0.10^a
Container and closure	05	4.68±0.03^a	4.68±0.03^a	4.68±0.03^a	4.68±0.03^a
Total score	100	93.37±0.42^a	92.96±0.42^a	92.61±0.43^a	92.55±0.43^a

Data are presented as means±SEM (n=30).
$^{a-b}$Means with same superscript in a row do not vary significantly (P<0.05) from each other.

Umbelino *et al*. (2001) also showed that the addition of iron (microencapsulated ferrous sulphate) do not interfere in the sensory properties of soy yoghurt. Achanta *et al*. (2007) reported that non significant (P>0.05) differences was observed for flavour and appearance scores of the yoghurts fortified with the iron (ferric orthophosphate) compared to the control. Umbelino and Rossi (2009) reported that the addition of iron (microencapsulated ferrous sulphate@12 ppm iron) did not significantly affect the sensory scores for all evaluated attributes. Azzam (2009) reported that the source of iron used for fortification had non significant effect on smoothness of yoghurt samples when fresh and during storage.

It is well known that major off-flavours may be associated with fortified dairy milk products: oxidised flavour resulting from catalysis of lipid oxidation by iron and metallic flavour contributed by iron salts (Jackson and Lee 1992). No oxidative rancidity was detected in fresh yoghurt and during storage samples (Abd-Rabou *et al*. 1999; Osman and Ismail 2004) while, Mehanna *et al*. (2000) concluded that fresh yoghurt fortified with iron from different sources seem to be slightly affected by iron concentration.

El-Kholy *et al.* (2011) reported that the sensory quality of iron-fortified dairy foods has been shown to be affected by the type of iron salt used. They showed that the highest flavour score was for control and yoghurt fortified with iron-whey protein chelate (Fe-WP) when fresh and during storage. The lowest score was for yoghurt fortified with ammonium ferrous sulphate which showed a slight metallic flavour. Also, the iron treatments did not affect significantly the body and texture of yoghurt. The colour and appearance of yoghurt fortified with iron was not affected by the sources of iron used (ammonium ferric sulphate, ammonium ferrous sulphate, iron-casein complex and iron-whey protein complex). The total score of yoghurt fortified with iron from different sources showed that the addition of Fe-WP resulted in yoghurt quite similar to that of control yoghurt.

4.17.2 Effect of milk fortification on pH, acidity, water holding capacity (WHC) and syneresis of yoghurt

Appropriate acid concentration is one the important factors ensuring quality of fermented milk. There was a slight but statistically non significant difference in pH, acidity, water holding capacity and syneresis of yoghurt samples made from control and fortified milk (table 4.93). However, pH and syneresis of fortified ones being slightly lower than the control samples. Acidity and water holding capacity of fortified samples was slightly higher than that of control samples.

Our results were in accordance with El-Behairy and Mohamed (2010) who reported a decrease in pH and increase in acidity of yoghurt samples prepared from iron fortified milk compared to those prepared from control milk was observed. El-Kholy *et al.* (2011) reported that the changes in the pH values for all yoghurt treatments with iron (Fe-WP complex) were not significantly ($p<0.05$) different.

However, Umbelino and Rossi (2009) reported that the initial titratable acidity of control soy yoghurt (0.87±0.01% lactic acid) was higher than of fortified product (0.85±0.01% lactic acid). They also showed that unfortified (control) and iron fortified soy yoghurt required 4.5 and 5.15 h to reduce pH to the 4.4-4.5 range, respectively, indicating that the starter culture was capable to produce enough acids in a suitable time period and that the fortification do not make the fermentation process unviable.

Our findings were in accordance with Achanta *et al.* (2007) who studied the rheological properties of yoghurts fortified with various minerals and reported that the mean syneresis values of yoghurts fortified with iron (ferric orthophosphate) were significantly ($P<0.05$) lower than in the control yoghurt. They reported that yoghurt fortified with iron had better water-holding capacities compared to the control.

Gaucheron *et al.* (1997b) reported that there may be a change in casein structure when iron binds to micellar casein. This probable change in the structure of casein may explain better water holding capacity/less released whey in iron fortified yoghurts. El-Kholy *et al.* (2011) showed that there was non significant difference in syneresis among most of yoghurt fortified with different iron salts viz. ammonium ferric sulphate, ammonium ferrous sulphate, iron-casein complex and iron-whey protein complex.

Table 4.93: Effect of milk fortification on pH, acidity, WHC and syneresis of yoghurt

Sample	pH	Acidity (%)	WHC (%)	Syneresis (%)
Control	4.40 ± 0.02^a	0.879 ± 0.012^a	77.99 ± 0.54^a	3.33 ± 0.27^a
VA2500+FPP15	4.39 ± 0.01^a	0.879 ± 0.012^a	78.21 ± 0.51^a	3.30 ± 0.27^a
VA2500+FPP20	4.39 ± 0.02^a	0.892 ± 0.020^a	78.30 ± 0.56^a	3.27 ± 0.24^a
VA2500+FPP25	4.38 ± 0.01^a	0.902 ± 0.021^a	78.63 ± 0.68^a	3.17 ± 0.30^a
VA2500+FG15	4.39 ± 0.02^a	0.886 ± 0.016^a	78.27 ± 0.54^a	3.23 ± 0.27^a
VA2500+FG20	4.38 ± 0.02^a	0.899 ± 0.023^a	78.54 ± 0.67^a	3.13 ± 0.27^a
VA2500+FG25	4.38 ± 0.02^a	0.916 ± 0.024^a	78.81 ± 0.77^a	3.07 ± 0.30^a

Data are presented as means±SEM (n=3).
$^{a-b}$Means with same superscript in a column do not vary significantly ($P<0.05$) from each other.

4.17.3 Effect of milk fortification on acetaldehyde content, setting time and *Streptococcus thermophilus* and *Lactobacillus bulgaricus* count of yoghurt

The compounds which impart the distinctive flavour to yoghurt are lactic acid and a variety of volatile organic aroma compounds, produced by *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (Beshkova *et al.* 1998). Gallardo-Escamilla *et al*. (2005) reported that the volatile compounds such as acetaldehyde and diacetyl are a key compound for typical yoghurt aroma. Acetaldehyde is recognised as a major flavour component in yoghurt (Bottazzi *et al*. 1973; Dumont and Adda 1973; Law 1981). Therefore, acetaldehyde content of yoghurt samples (control and fortified samples) was determined to evaluate the effect of iron fortification on flavour producing activity of yoghurt cultures.

There was a slight but statistically non significant difference ($P<0.05$) in acetaldehyde content, setting time, *Streptococcus thermophilus* and *Lactobacillus bulgaricus* count of yoghurt samples made from control and fortified milk (table 4.94). Acetaldehyde content of fortified samples was slightly higher than the control samples. Control samples took slightly more time to set and form a firm coagulum than fortified samples. The results showed that, there was a decrease in setting time on addition of iron to milk, however, the values were non significantly different ($P<0.05$) from each other. *Streptococcus thermophilus* and *Lactobacillus bulgaricus* count of yoghurt prepared from fortified milk samples also showed non significant difference ($P<0.05$), however, slight increase was observed for both *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in fortified yoghurt samples compared to control samples.

El-Kholy *et al*. (2011) showed that there were non significant ($P<0.05$) differences in acetaldehyde among most iron treatments. Our results were in accordance with El-Behairy and Mohamed (2010) who reported higher lactic acid count in yoghurt prepared from iron fortified milk (ferric chloride and ferrous sulphate). However, Simova *et al.* (2008) reported that iron fortification of milk with ferrous lactate @8, 15 and 27 ppm iron had no effect on the incubation time required for the yoghurt mixes to reach pH 4.5±0.1 and it did not affect significantly the growth and viability of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* during milk fermentation and storage of yoghurt. Lactic acid bacteria are reported to be exception among living organisms in that they show no iron requirements (Bruyneel *et al*. 1989; Cervaux *et al*. 2000; Weinberg 1997). There are some reports that some *Lactobacillus* species have the ability to bind ferric hydroxide at their cell surface, rendering it unavailable to pathogenic microorganisms.

Hekmat and McMahon (1997) also reported that **c**ounts of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* after 1 day of storage in iron-fortified skim yoghurts (casein chelated iron and whey protein-chelated iron@20 ppm) were not significantly different from numbers in unfortified yoghurts. Iron fortification had no effect on the incubation time required for the yoghurt mixes to reach pH 4.3. El-Kholy *et al*. (2011) showed that fortification of yoghurt with different iron salts had no effect on the total lactic acid bacteria in all treatments. Ocak and Köse (2010) found that iron fortification with ferric nitrate (@0.3 and 0.6 ppm) had no effect on the incubation time required for the yoghurt mixes to reach pH 4.5 ± 0.1.

Table 4.94: Effect of milk fortification on acetaldehyde content, setting time and *Streptococcus thermophilus* and *Lactobacillus bulgaricus* count of yoghurt

Sample	**Acetaldehyde (mg/g)**	**Setting time (hrs)**	***Strep. thermophilus*** **(10^6 cfu/ml)**	***Lb. bulgaricus*** **(10^6 cfu/ml)**
Control	1.368 ± 0.028^a	4.83 ± 0.15^a	586.33 ± 12.81^a	527.67 ± 8.41^a
VA2500+FPP15	1.369 ± 0.029^a	4.80 ± 0.15^a	591.00 ± 13.20^a	529.33 ± 8.95^a
VA2500+FPP20	1.370 ± 0.028^a	4.73 ± 0.15^a	595.33 ± 13.91^a	533.67 ± 9.82^a
VA2500+FPP25	1.375 ± 0.028^a	4.67 ± 0.12^a	603.00 ± 16.17^a	538.00 ± 9.82^a
VA2500+FG15	1.369 ± 0.029^a	4.73 ± 0.15^a	593.30 ± 13.91^a	532.30 ± 9.26^a
VA2500+FG20	1.370 ± 0.0292^a	4.67 ± 0.18^a	599.00 ± 14.05^a	538.00 ± 10.12^a
VA2500+FG25	1.375 ± 0.029^a	4.57 ± 0.15^a	606.00 ± 16.17^a	541.67 ± 10.60^a

Data are presented as means±SEM (n=3).
$^{a\text{-}b}$Means with same superscript in a column do not vary significantly ($P<0.05$) from each other.

4.17.4 Effect of milk fortification on viscosity of yoghurt

Viscosity of yoghurt prepared from fortified milk sample was slightly more than the control yoghurt. At 10, 20 and 30 rpm, viscosity of all the samples showed statistically non significant difference ($P<0.05$) from each other (table 4.95). Our results were in accordance with Achanta *et al*. (2007) who reported that there was a slight but non significant increase in viscosity of yoghurt samples fortified with iron (ferric orthophosphate). Umbelino *et al*. (2001) reported that addition of iron did not alter the viscosity of soy yoghurt. Umbelino and Rossi (2009) reported that storage did not have any significant effect on viscosity and consistency of the soy yoghurt fortified or not with iron (microencapsulated ferrous sulphate). The average consistency did not vary between the samples. However, viscosity of iron soy yoghurt was lower than control soy yoghurt ($p<0.05$), during all the storage period. Their results revealed that, despite the differences in rheological properties, the control and iron fortified soy yoghurt presented a stable gel with characteristic of natural set yoghurt. Rice and McMahon (1998) reported that there was no effect of iron on the apparent viscosity of mozzarella cheese fortified with iron at 25 mg/kg.

Table 4.95: Effect of milk fortification on viscosity of yoghurt

Sample	Viscosity at 10 rpm (cP)	Viscosity at 20 rpm (cP)	Viscosity at 30 rpm (cP)
Control	3545.65±46.73^{a}	2070.70±43.61^{a}	1370.60±57.06^{a}
VA2500+FPP15	3547.62±46.74^{a}	2073.14±43.38^{a}	1374.33±57.34^{a}
VA2500+FPP20	3554.69±45.64^{a}	2082.90±43.27^{a}	1396.32±55.68^{a}
VA2500+FPP25	3567.58±48.20^{a}	2105.92±38.15^{a}	1455.44±38.16^{a}
VA2500+FG15	3549.31±46.20^{a}	2079.18±44.50^{a}	1376.17±57.67^{a}
VA2500+FG20	3567.30±44.27^{a}	2120.75±38.72^{a}	1423.22±53.48^{a}
VA2500+FG25	3587.75±41.28^{a}	2133.99±35.14^{a}	1470.64±39.26^{a}

Data are presented as means±SEM (n=3).
$^{a-b}$Means with same superscript in a column do not vary significantly ($P<0.05$) from each other.

4.17.5 Effect of milk fortification on texture profile of yoghurt

It is evident from table 4.96 that control and fortified yoghurt samples showed non significant difference ($P<0.05$) in firmness except VA2500+FG25 yoghurt sample, however, VA2500+FG25 sample did not differ significantly from VA2500+FG20. In terms of work of adhesion, all fortified samples showed non significant difference ($P<0.05$) from control yoghurt except VA2500+FPP25, VA2500+FG20 and VA2500+FG25. There was non significant difference ($P<0.05$) observed in work of shear for all yoghurt samples. VA2500+FPP25, VA2500+FG20 and VA2500+FG25 differed significantly from control yoghurt in terms of stickiness.

Our results were in consonance with Ocak and Köse (2010) who reported that addition of iron (ferric nitrate@0.3 and 0.6 ppm) to milk caused a general increase in firmness and cohesiveness of yoghurt in comparison to the control yoghurt and this was found statistically significant ($P<0.01$). Sandoval-Castilla *et al.* (2004) stated that the protein matrix was responsible for the firmness and springiness of yoghurt.

Table 4.96: Effect of milk fortification on texture profile of yoghurt

Sample	Firmness N	Work of adhesion N.s	Work of shear N.s	Stickiness N
Control	1.942±0.029^{a}	-1.761±0.036^{a}	15.565±0.337^{a}	-0.280±0.012^{a}
VA2500+FPP15	1.946±0.029^{a}	-1.754±0.038^{a}	15.571±0.337^{a}	-0.268±0.010^{a}
VA2500+FPP20	1.956±0.032^{a}	-1.714±0.039^{a}	15.644±0.338^{a}	-0.255±0.012^{ab}
VA2500+FPP25	2.003±0.044^{a}	-1.567±0.046^{c}	15.813±0.338^{a}	-0.244±0.014^{bc}
VA2500+FG15	1.952±0.029^{a}	-1.734±0.039^{a}	15.582±0.334^{a}	-0.258±0.013^{ab}
VA2500+FG20	1.975±0.029^{ab}	-1.676±0.037^{b}	15.663±0.334^{a}	-0.242±0.014^{bc}
VA2500+FG25	2.026±0.038^{b}	-1.497±0.049^{c}	15.856±0.337^{a}	-0.230±0.015^{c}

Data are presented as means±SEM (n=3).
$^{a-b}$Means with same superscript in a column do not vary significantly ($P<0.05$) from each other.

The above results for *dahi* and yoghurt suggest possibility of making good quality *dahi* and yoghurt by fortifying milk with vitamin A@2500 IU/L and FPP soluble or vitamin A and FG hydrate@25 ppm, the resultant *dahi* and yoghurt did not differ from the control and did not show any inhibition of starter bacteria or any defects in the organoleptic properties.

4.18 Bioavailability of added micronutrients in the fortified milk

4.18.1 *In vitro* digestibility of iron and vitamin A

Data on the bioavailability of micronutrients (endogenous or synthetic) from dairy products in humans are very limited (Russel *et al*. 2001; de Jong *et al*. 2005; Herrero-Barbudo *et al*. 2009). *In vitro* models based on human physiology have been developed as simple, inexpensive and reproducible tools to predict the bioavailability of different food components (i.e. ascorbic acid, carotenoids and polyphenols) (Failla and Chitchumroonchokchai 2005) although their potential predictive value regarding absorption in humans should be validated in different *in vivo* situations (Oomen *et al*. 2003).

Using an *in vitro* approach, the amount of a food component that is released from the food matrix is commonly referred to as “bioaccessibility’’. In addition, fat soluble components must be incorporated into mixed micelles before absorption and; considering that the content in this phase and residues reflect the amounts of compounds available in the small and large intestine respectively, the efficiency of micellisation (quantities transferred into the aqueous-micellar fraction) is used as an estimate of the relative bioavailability (Failla and Chitchumroonchokchai 2005). The application of *in vitro* digestion models may be of relevance in understanding the bioavailability of phytochemicals in humans, as well as in the food industry, including food technology, functional foods and product design (Granado *et al*. 2006).

In vitro bioaccessibility of iron and vitamin A in control and fortified milk samples was determined under simulated gastro-intestinal conditions. In case of iron, % bioaccessibility of iron in fortified milk was not significantly different ($P<0.05$) from control and amongst the fortified samples as shown in table 4.97. Bioaccessibility of milk samples fortified with vitamin A+iron was slightly higher than that of milk fortified with iron alone. This increase can be related to the effect of vitamin A on solubility of iron salts. Our results were in accordance with Garcia-Casal *et al*. (2003) who reported that vitamin A helps in enhancing solubility of iron at pH 2 and 6 and inclusion of vitamin A in diet was able to avoid precipitation of half of the iron. They also reported that addition of vitamin A produced 1.8 times increment in iron absorption, which might be due to the stabilising effect of vitamin A on iron. In case of vitamin A, all the milk samples showed non significant difference ($P<0.05$) in terms of % bioaccessibility (table 4.98), suggesting that the bioaccessibility of all samples (control and fortified) was comparable under simulated gastrointestinal conditions.

Herrero-Barbudo *et al*. (2009) assessed the applicability of an *in vitro* digestion model to estimate the bioaccessibility of fat soluble vitamins (vitamin A and E) contained in commercially available fortified liquid milk and showed that the *in vitro* protocol was useful in assessing the stability, the degree of hydrolysis of ester forms and micellarisation of vitamin A from liquid milk. Overall, *in vitro* results were consistent with *in vivo* observations supporting the potential applicability and predictive value of the *in vitro* approach to assess the bioavailability of fat soluble vitamins from dairy matrices.

Miller *et al*. (1981) showed that *in vitro* method accurately reflected the actual relative bioavailability of iron for a series of meals viz. eggs, orange juice, tea, coffee, cola or whole wheat bread. In particular, the *in vitro* procedures have been shown to provide availability measurements that correlate well with *in vivo* studies (Miller and Berner 1989). The results of *in vitro* method used by Argyri *et al*. (2009) for prediction of iron bioavailability on the basis of the percent dialysable ferrous or total iron were in accordance with Hallberg *et al*. (1986) who reported the effect of enhancers and inhibitors on iron absorption. The *in vitro* method is a rapid, simple and cheap alternative to *in vivo* methods for measuring bioavailability of iron in milk and dairy products (Perez *et al*. 2002).

Table 4.97: *In vitro* bioaccessibility of iron

Sample	**% Bioaccessibility**
Control milk	37.22 ± 1.84^{a}
Milk+FPP25	36.96 ± 1.92^{a}
Milk+FG25	36.91 ± 1.66^{a}
Milk+VA2500+FPP25	37.35 ± 1.91^{a}
Milk+VA2500+FG25	37.23 ± 1.73^{a}

Data are presented as means±SEM (n=3).
$^{a\text{-}b}$Means with same superscript in a column do not vary significantly ($P<0.05$) from each other.

Table 4.98: *In vitro* bioaccessibility of vitamin A

Sample	**% Bioaccessibility**
Control milk	77.66 ± 1.50^{a}
Milk+VA2500	77.33 ± 0.84^{a}
Milk+VA2500+FPP25	77.38 ± 0.89^{a}
Milk+VA2500+FG25	77.50 ± 1.10^{a}

Data are presented as means±SEM (n=3).
$^{a\text{-}b}$Means with same superscript in a column do not vary significantly ($P<0.05$) from each other.

4.18.2 Iron and vitamin A bioavailability (*in vivo* studies)

It has been recommended that the use of multiple iron status indicators (i.e. plasma ferritin, total iron binding capacity (TIBC), unsaturated iron binding capacity (UIBC), transferrin saturation and haemoglobin) simultaneously provide a more accurate measure of iron status than any single index (Cook *et al*. 1976; Looker *et al*. 1995; Kasdan 1996).

Therefore, depending on the facilities available, we selected balance studies, haemoglobin, plasma ferritin, plasma transferrin and liver storage of iron for having an estimate about the body's response towards iron. For vitamin A, balance studies and vitamin A status of liver were used to assess its bioavailability *in vivo*.

Among the two iron salts, ferric pyrophosphate (FPP) soluble instead of ferrous gluconate (FG) hydrate was chosen for conducting animal study, depending on the comparative effect of the two iron salts (FPP soluble and FG hydrate) as shown in previous experiments. It is clear from the results of previous experiments that FG hydrate being a freely water soluble and unprotected salt of iron in the form of ferrous ion caused greater changes in the milk system and affected inherent and added constituents of milk more compared to FPP soluble which is a freely water soluble, chelated salt of iron (ferric form), caused lesser changes in matrix due to its restricted interaction with matrix and other constituents as suggested by its structure (iron core is tightly chelated to sodium citrate and pyrophosphate). In the animal study results, iron salt used will be referred to as FPP soluble and vitamin A will be referred to as vitamin A acetate.

For conducting the animal trials, toned milk was fortified with FPP soluble alone (@25 ppm) and FPP soluble (@25 ppm)+vitamin A acetate (@2500 IU/L) and lyophilised to convert it into powder. Lyophilates of the unfortified, FPP soluble fortified and FPP soluble+vitamin A acetate fortified milk were mixed with the basal diet in the ratio of (1:2) and were given to rats *ad-libitum*. The rats consumed ~10-15 g diet daily. Unfortified lyophiltaes+basal diet (A group) contained 1.201 µg vitamin A and 0.48 mg iron per 15 g diet; FPP soluble fortified lyophilates+basal diet (B group) contained 1.201 µg vitamin A and 6.73 mg iron per 15 g diet; and FPP soluble+vitamin A acetate fortified lyophilates+basal diet (C group) contained 207.25 µg vitamin A and 6.73 mg iron per 15 g diet.

To study the iron bioavailability, haemoglobin content of blood, ferritin content and transferrin content of blood plasma of control and anaemic group rats were analysed at 0 day and 21st day and compared statistically between control and anaemic groups; and among unfortified group, iron fortified and vitamin A +iron fortified group.

4.18.2.1 Weight of control group rats

It is clear from table 4.99 that there was non significant difference in % increase in weight of rats of control group A (unfortified) and control group C (vitamin A+iron fortified) after 21 days, however, % increase in weight of control group B rats (iron fortified) was significantly different from other two groups ($P<0.05$). The results indicated that no correlation was found between intake of iron and vitamin A and increase in body weight of rats under study.

Table 4.99: Weight of control group rats

Group	Weight on 0 day (g)	Weight after 21 days (g)	% ↑ in weight from 0 day
Control A	59.91±4.76	146.10±8.38	147.38 ± 8.19^{a}
Control B	76.65±5.32	166.03±5.90	120.60 ± 9.39^{b}
Control C	68.99±6.00	165.53±9.33	144.08 ± 8.03^{a}

Data are presented as means±SEM (n = 8).
$^{a\text{-}b}$Means with same superscript in a column do not vary significantly ($P<0.05$) from each other.

4.18.2.2 Weight of anaemic group rats

There was non significant difference in % increase in weight after 21 days of rats of anaemic group A (unfortified) and anaemic group B (iron fortified), however, % increase in weight of anaemic group C rats (iron+VA fortified) was significantly different from anaemic group A (unfortified), but not from anaemic B group (iron fortified) ($P<0.05$). However, no correlation was found between intake of iron and vitamin A and % increase in body weight of anaemic rats under study (table 4.100).

Table 4.100: Weight of anaemic group rats

Group	Weight on 0 day PEP (g)	Weight on 0 day EP(g)	% ↑ in weight from PEP	Weight after 21 days (g)	% ↑ in weight from 0 day of EP
Anaemic A	77.38±6.39	157.28±8.88	106.43±6.47	212.16±7.34	66.16 ± 5.74^{a}
Anaemic B	81.64±3.90	162.26±5.60	100.10±5.25	212.48±7.41	69.91 ± 6.68^{ab}
Anaemic C	89.38±2.89	175.66±7.37	96.35±4.34	237.83±8.00	82.06 ± 6.70^{b}

Data are presented as means±SEM (n=8).
$^{a\text{-}b}$Means with same superscript in a column do not vary significantly ($P<0.05$) from each other.
PEP=pre-experimental period
EP=experimental period

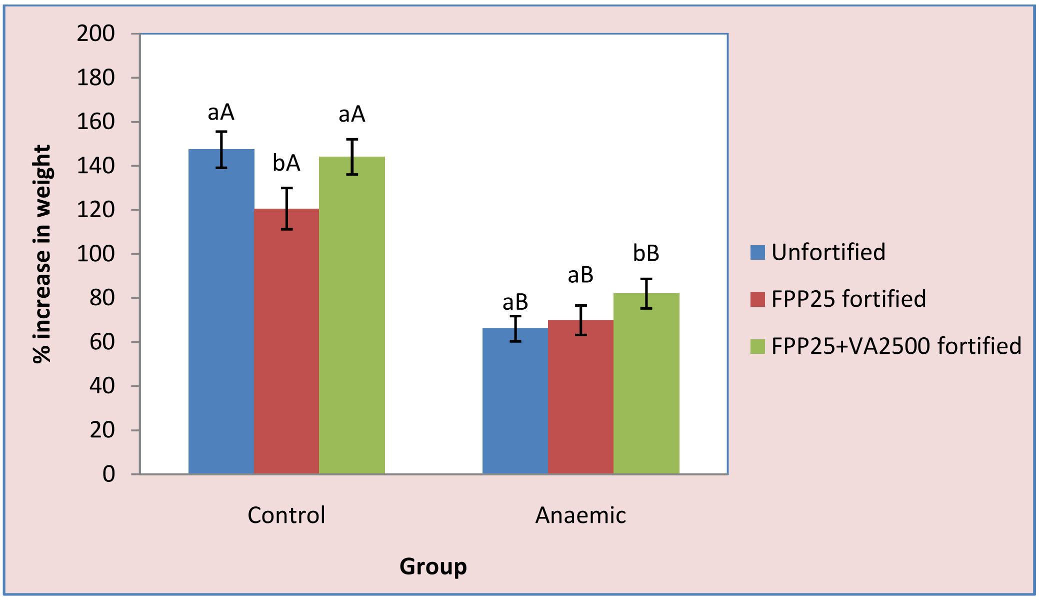

Figure 4.40: Percentage increase in weight of control and anaemic group rats

$^{a-b}$Different lowercase letters denote significant difference (P<0.05) across sub-groups (unfortified, FPP25 fortified and VA2500+FPP25 fortified).

$^{A-B}$Different uppercase letters denote significant difference (P<0.05) between groups (control and anaemic).

Error bars show the variations of eight determinations in terms of standard error of mean.

Two way ANOVA (P<0.05) revealed that there was significant difference in terms of body weight gain between control and anaemic groups. In control group, there was significant difference between iron fortified group and other two subgroups i.e. control unfortified and control vitamin A+iron fortified. In anaemic group, rats fed with vitamin A+iron fortified diet differed significantly from other two groups. However, no correlation was observed between iron and vitamin A intake and iron status of body of animals under study (figure 4.40)

Burri and Jacob (1988) observed that there was non significant difference in body weight among the rats fed on diets containing different levels of vitamin A suggesting that vitamin A had no influence on weight gain. Haro-Vicente *et al.* (2008) reported that there was non significant effect on weight gains of feeding the rats with different sources of iron. Ranhotra *et al.* (1981) reported that the body weight gain of rats on different diets (iron deficient and normal diet) did not differ significantly.

However, Hunter (1978) reported that difference in growth of rats fed with iron-adequate (control) and iron deficient rats was apparent after the first week. After 5 weeks, the control rats were about 50 g heavier than the iron-deficient rats. When deficient rats were fed a diet containing adequate iron their growth increased markedly during the first week of iron repletion. Body weights of iron-deficient rats approached those of control rats after 4 to 5 weeks of iron repletion. These differences could be due to due to strain variation, climatic conditions and other secondary factors.

4.18.2.3 Biological indices

Biological indices, namely apparent digestibility coefficient, balance-retention and percentage retention were calculated in order to check the digestive and metabolic utilisation of iron and vitamin A. The biological indices as observed during the experimental period are recorded in table 4.101, 4.102, 4.103 and 4.104.

4.18.2.3.1 Iron balance studies of control group rats

Apparent digestibility coefficient of iron for control group rats was significantly different ($P<0.05$) for all the groups (36.34, 37.44 and 39.93% for control A group, control B group and control C group, respectively). Similarly, % retention/intake of iron for control group rats was also significantly different ($P<0.05$) for all the groups (35.96, 36.85 and 39.29% for control A group, control B group and control C group, respectively (table 4.101).

Table 4.101: Iron balance studies of control group rats

Group→ Parameters↓	Control A	Control B	Control C
Food intake (g/d)	10.37±0.14	11.13±0.23	11.73±0.12
Iron intake (mg/d)	0.33±0.01	5.00±0.11	5.27±0.06
Faecal excretion (mg/d)	0.21±0.003	3.12±0.070	3.17±0.040
Absorbed iron (mg/d)	0.121±0.002	1.872±0.038	2.101±0.025
Apparent digestibility coefficient (%)	36.34±0.12[a]	37.44±0.12[b]	39.93±0.38[c]
Urinary Iron (mg/d)	0.0013±0.0001	0.0294±0.0011	0.0336±.0019
Iron balance (mg/d)	0.119±0.002	1.842±0.038	2.068±0.025
% Retention/Intake	35.96±0.12[a]	36.85±0.12[b]	39.29±0.39[c]

Data are presented as means±SEM (n=8).
[a-b]Means with same superscript in a row do not vary significantly (P<0.05) from each other.

4.18.2.3.2 Iron balance studies of anaemic group rats

Apparent digestibility coefficient of iron for anaemic group rats was significantly different (P<0.05) for all the groups (38.86, 40.98 and 43.07% for anaemic A group, anaemic B group and anaemic C group, respectively) as shown in table 4.102. Similarly, % retention/intake of iron for anaemic group rats was also significantly different (P<0.05) for all the groups (38.64, 40.50 and 42.53% for anaemic A group, anaemic B group and anaemic C group, respectively. The results indicated that % apparent digestibility coefficient and % retention/intake of anaemic B (iron fortified) group were greater than anaemic A (unfortified) and those of anaemic C (iron+VA fortified) group were higher than anaemic B group, which indicate that vitamin A might have played role in enhancement of iron absorption.

Table 4.102: Iron balance studies of anaemic group rats

Group→ Parameters↓	**Anaemic A**	**Anaemic B**	**Anaemic C**
Food intake (g/d)	13.93±0.24	14.40±0.27	14.65±0.15
Iron intake (mg/d)	0.446±0.008	6.465±0.123	6.578±0.069
Faecal excretion (mg/d)	0.273±0.005	3.815±0.072	3.745±0.043
Absorbed iron (mg/d)	0.173±0.003	2.649±0.052	2.833±0.027
Apparent digestibility coefficient (%)	38.86±0.16^{a}	40.98±0.08^{b}	43.07±0.10^{c}
Urinary iron (mg/d)	0.0012±0.0004	0.0327±0.0004	0.035±0.0011
Iron balance (mg/d)	0.172±0.003	2.618±0.051	2.798±0.028
% Retention/Intake	38.64±0.16^{a}	40.50±0.08^{b}	42.53±0.11^{c}

Data are presented as means±SEM (n=8).
$^{a-b}$Means with same superscript in a row do not vary significantly (P<0.05) from each other.

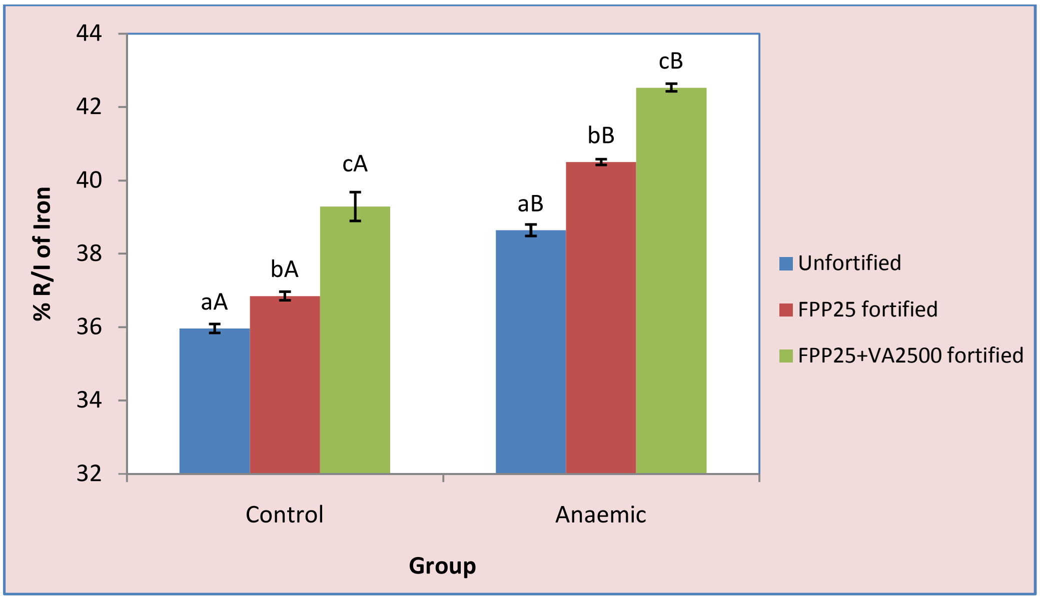

Figure 4.41: Retention/intake (%) of iron for control and anaemic group rats
$^{a-b}$Different lowercase letters denote significant difference (P<0.05) across sub-groups (unfortified, FPP25 fortified and VA2500+FPP25 fortified).
$^{A-B}$Different uppercase letters denote significant difference (P<0.05) between groups (control and anaemic).
Error bars show the variations of eight determinations in terms of standard error of mean.

Two way ANOVA (P<0.05) for the effect of diet and anaemia on iron absorption and retention in body revealed that % retention/intake was found to be higher and significantly different for all anaemic groups compared to control group rats and in both the groups, rats fed with vitamin A supplemented diet showed significantly higher iron absorption than those fed with control or only iron fortified diet as shown in figure 4.41.

The apparent digestibility coefficient, iron balance and % retention/intake of iron were greater for the iron-deficient rats than for their controls, regardless of the type of diet consumed. These parameters were increased by anaemia and also by the diet. Higher levels were obtained for anaemic rats in comparison with the controls, for all the diets studied. Our results were in accordance with Alferez *et al*. (2006) who reported that the digestive utilisation of iron was greater in anaemic rats than in their controls, regardless of the type of diet consumed. This revealed that there was a greater avidity for iron among anaemic rats, which might be because ferropenic anaemia increased the number of membrane receptors affected by non-heme iron absorption and thus increases iron absorption (Forellat *et al.* 2000). The increase in the apparent digestibility coefficient percentage of iron in deficient rats was similar to that reported by Pallare´s *et al*. (1993). Iron balance and the % retention/intake were also greater in anaemic rats than in their controls for all the three diets studied.

Comparison of the effects of the three diets revealed that among the controls and the iron-deficient rats, % apparent digestibility coefficient, iron balance and % retention/intake of control B (iron fortified) group were greater than control A (unfortified) and those of control C (iron+VA fortified) group were higher than control B group, which indicated that improvement in iron absorption could be due to inclusion of vitamin A in diet. On the same lines, iron absorption was enhanced on addition of vitamin A as presented in the results for *in vitro* studies (section 5.20.1). The increased absorption of iron in presence of vitamin A could be related to the increased solubility of iron salts in the gut at pH 2 and 6 when vitamin A was included in diet.

Our results were in corroboration with Gracia-Casal *et al*. (2003) who also reported that addition of vitamin A produced a 1.8 times increment in iron absorption and reported that addition of vitamin A improved iron absorption by a process at least partially due to the stabilising effect of this vitamin on iron. However, Bloem (1995) suggested that vitamin A influences iron metabolism, but this mechanism has not yet been fully elucidated. They reported that it is unlikely for vitamin A to interfere directly with the intestinal absorption of iron, but it may mobilise available iron stores and use them to form Hb. Vitamin A deficiency impaired iron mobilisation from stores and had little influence on iron absorption (Mejia *et al*. 1979; Amine *et al*. 1970).

4.18.2.3.3 Vitamin A balance studies of control group rats

Apparent digestibility coefficient of vitamin A, unlike in the *in vitro* studies for control group rats was significantly different ($P<0.05$) for all the groups (63.42, 67.39 and 85.87% for control A group, control B group and control C group, respectively) as shown in table 4.103. Similarly, % retention/intake of vitamin A for control group rats was also significantly different ($P<0.05$) for all the groups (62.34, 63.84 and 85.80% for control A group, control B group and control C group, respectively. The results indicated that % apparent digestibility coefficient and % retention coefficient of control B (iron fortified) group were greater than control A (unfortified) and those of control C (iron+VA fortified) group were higher than control B group, which indicated that increase in absorption of vitamin A might be due to effect of iron on vitamin A passage through intestinal epithelium.

Table 4.103: Vitamin A balance studies of control group rats

Group→ Parameters↓	**Control A**	**Control B**	**Control C**
Food intake (g/d)	10.37±0.14	11.13±0.23	11.73±0.12
VA intake (µg/d)	0.83±0.01	0.89±0.02	162.23±1.71
Faecal excretion (µg/d)	0.304±0.005	0.29±0.007	23.043±0.915
Absorbed VA (µg/d)	0.526±0.009	0.6±0.012	139.183±1.985
Apparent digestibility coefficient (%)	63.42±0.40^{a}	67.39±0.28^{b}	85.87±0.60^{c}
Urinary VA (µg/d)	0.0089±0.0019	0.0314±0.0039	0.104±0.0264
VA balance (µg/d)	0.597±0.009	0.657±0.019	155.825±3.098
% Retention/Intake	62.34±0.57^{a}	63.84±0.60^{b}	85.80±0.60^{c}

Data are presented as means±SEM (n=8).
$^{a-b}$Means with same superscript in a row do not vary significantly ($P<0.05$) from each other.

4.18.2.3.4 Vitamin A balance studies of anaemic group rats

Apparent digestibility coefficient of vitamin A for anaemic group rats was significantly different ($P<0.05$) for all the groups (67.29, 72.93 and 92.49% for anaemic A group, anaemic B group and anaemic C group, respectively) as shown in table 4.104. Similarly, % retention/intake of vitamin A for anaemic group rats was also significantly different ($P<0.05$) for all the groups (66.64, 68.54 and 92.45% for anaemic A group, anaemic B group and anaemic C group, respectively. The results indicated that % apparent digestibility coefficient, iron balance and % retention/intake of anaemic B (iron fortified) group were greater than anaemic A (unfortified) and those of anaemic C (iron+VA fortified) group were higher than anaemic B group, which indicated that iron might have played a pivotal role in improving absorption of vitamin A.

Table 4.104: Vitamin A balance studies of anaemic group rats

Group→ Parameters↓	**Anaemic A**	**Anaemic B**	**Anaemic C**
Food intake (g/d)	13.93±0.24	14.40±0.27	14.65±0.15
VA intake (μg/d)	1.115±0.019	1.152±0.022	202.604±2.127
Faecal excretion (μg/d)	0.37±0.01	0.31±0.01	15.17±0.68
Absorbed VA (μg/d)	0.75±0.01	0.84±0.02	187.43±2.53
Apparent digestibility coefficient (%)	67.29±0.23^{a}	72.93±0.18^{b}	92.49±0.38^{c}
Urinary VA (μg/d)	0.0072±0.0004	0.0506±0.002	0.0754±0.0039
VA balance (μg/d)	0.743±0.012	0.789±0.017	187.357±2.524
% Retention/Intake	66.64±0.23^{a}	68.54±0.26^{b}	92.45±0.38^{c}

Data are presented as means±SEM (n=8).
$^{a-b}$Means with same superscript in a row do not vary significantly ($P<0.05$) from each other.

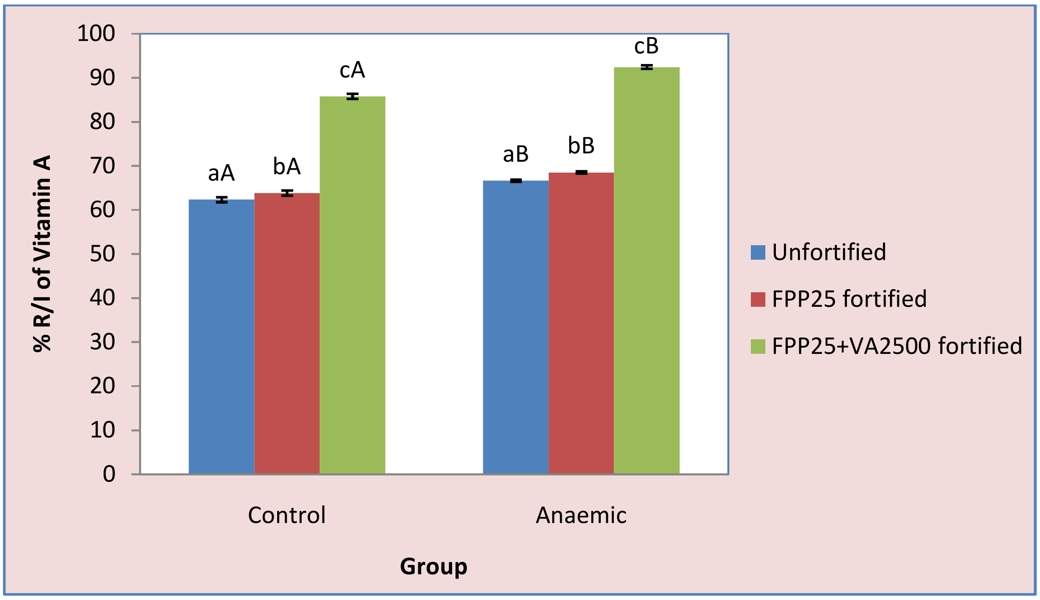

Figure 4.42: Retention/intake (%) of vitamin A for control and anaemic group rats.

$^{a-b}$Different lowercase letters denote significant difference (P<0.05) across sub-groups (unfortified, FPP25 fortified and VA2500+FPP25 fortified).
$^{A-B}$Different uppercase letters denote significant difference (P<0.05) between groups (control and anaemic).
Error bars show the variations of eight determinations in terms of standard error of mean.

Two way ANOVA (P<0.05) for the effect of diet and anaemia on vitamin A absorption and retention in body revealed that % retention/intake for vitamin A was found to be higher and significantly different for all anaemic groups compared to control group rats and in both the groups, rats fed with iron supplemented diet showed significantly higher vitamin A absorption than those fed with control diet as shown in figure 4.42.

Apparent digestibility coefficient and retention coefficient of vitamin A was found to be higher for all anaemic groups compared to control group rats. Iron deficiency might have caused change in rat physiology that might have led to increased absorption of vitamin A from gut. Studies on rats have shown that iron deficiency is associated with low plasma retinol concentration (Oliveira *et al.* 2008) which might have played role in increased absorption of vitamin A through intestinal epithelium. Iron affects the functioning of an enzyme retinyl ester hydrolase which helps in esterification of retinol and mobilisation from liver to body organs. Wieringa *et al.* (2007) reported that liver vitamin A store was greater in the vitamin A deficient rats which were supplemented with iron for 4-6 months. They suggested a possible redistribution of serum retinol and thus impairment in vitamin A deficiency observed after supplementation. Incorporation of iron in diet in the present study led to an increase in vitamin A absorption.

4.18.2.4 Haemoglobin repletion assay

Haemoglobin (Hb) repletion assay in rodents is considered to be a good predictor of iron bioavailability (Forbes *et al.* 1989). Haemoglobin estimation was done according to cyanomethaemoglobin method outlined by Drabkin and Austin (1932).

4.18.2.4.1 Haemoglobin content of control group rats

Haemoglobin content of control group rats was 13.67 g/dl blood and it increased to 13.96, 14.37, 14.87 for control A group, control B group and control C group, respectively after feeding with their respective diets for 21 days (table 4.105). % increase in haemoglobin content after 21 days feeding was 2.25, 5.22 and 8.90% for control A group, control B group and control C group, respectively. There was significant difference in haemoglobin content and % increase in haemoglobin of all the groups ($P<0.05$), being highest for control C group i.e. vitamin A+iron group suggesting that vitamin A helped in enhancing the utilisation of iron in body and its incorporation in haemoglobin. Our results were in accordance with those of Mejia and Chew (1988) kept the iron content of diet constant, varied vitamin A content and found that vitamin A supplementation significantly increased haemoglobin.

Table 4.105: Haemoglobin (Hb) content of control group rats

Group	Hb content 0 day (g/dl blood)	Hb content 21 days (g/dl blood)	% ↑ in Hb content from 0 day
Control A	13.67±0.16	13.96 ± 0.18^{a}	2.25 ± 1.16^{a}
Control B	13.67±0.16	14.37 ± 0.13^{b}	5.22 ± 1.03^{b}
Control C	13.67±0.16	14.87 ± 0.08^{c}	8.90 ± 1.30^{c}

Data are presented as means±SEM (n=8).

$^{a-b}$Means with same superscript in a column do not vary significantly ($P<0.05$) from each other.

4.18.2.4.2 Haemoglobin content of anaemic group rats

Anaemic group (24 rats) was subjected to a pre-experimental period of 21 days to induce anaemia. Haemoglobin content was within normal limits for the all the rats/animals used at the start and end of the experimental period. Haemoglobin content of anaemic rats was 13.41 g/dl at 0 day of pre-experimental period, which decreased to 8.25 g/dl after feeding the rats with low iron diet (anaemic diet) for 21 days. Haemoglobin content of anaemic rats increased to 10.93, 14.10 and 14.91 g/dl for anaemic A group, anaemic B group and anaemic C group, respectively after feeding with their respective diets for 21 days. % increase in haemoglobin after 21 days feeding was 32.75, 71.2 and 80.99% for anaemic A group, anaemic B group and anaemic C group, respectively (table 4.106). There was significant difference in haemoglobin content and percent increase in haemoglobin content of all the groups ($P<0.05$), being highest for anaemic C group i.e. vitamin A+iron group suggesting that vitamin A might have played role in enhancement of iron absorption and thus increase in haemoglobin content.

Demott (1971) reported increase in hematocrits of rats fed on iron fortified whole milk at 5.2 and 10.4 ppm concentrations of FPP soluble. Ranhotra *et al*. (1981) reported that bioavailability of a water-soluble iron citrate phosphate complex (potassium ferric pyrophosphate) was as high as ferrous sulphate and it was not affected by milk components.

Table 4.106: Haemoglobin (Hb) content of anaemic group rats

Group	Hb content PEP 0 day (g/dl blood)	Hb content 0 day EP (g/dl blood)	Hb content 21 days (g/dl blood)	% ↑ in Hb content from 0 day
Anaemic A	13.41±0.13	8.25±0.12	10.93±0.19^{a}	32.75±3.42^{a}
Anaemic B	13.41±0.13	8.25±0.12	14.10±0.14^{b}	71.20±3.23^{b}
Anaemic C	13.41±0.13	8.25±0.12	14.91±0.07^{c}	80.99±2.59^{c}

Data are presented as means±SEM (n=8).
$^{a-b}$Means with same superscript in a column do not vary significantly ($P<0.05$) from each other.
PEP=pre-experimental period
EP=experimental period

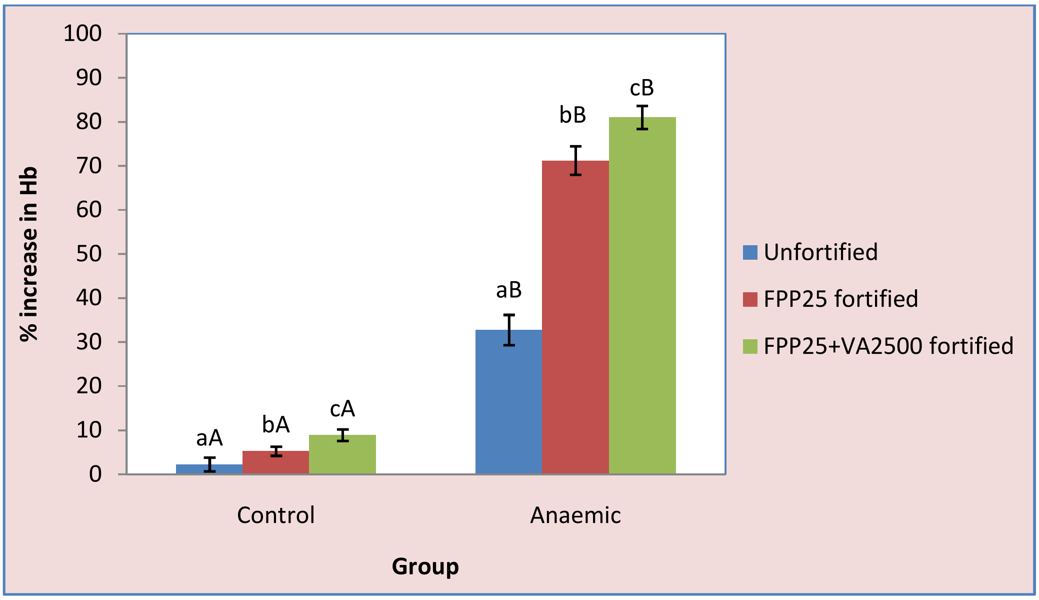

Figure 4.43: Percent increase in haemoglobin content in control and anaemic group rats

$^{a-b}$Different lowercase letters denote significant difference ($P<0.05$) across sub-groups (unfortified, FPP25 fortified and VA2500+FPP25 fortified).

$^{A-B}$Different uppercase letters denote significant difference ($P<0.05$) between groups (control and anaemic).

Error bars show the variations of eight determinations in terms of standard error of mean.

Two way ANOVA ($P<0.05$) revealed that the percentage increase in haemoglobin was affected by the diet and by anaemia. For the three diets assayed, % increase in haemoglobin in iron-deficient rats was higher and significantly different than among controls as shown in figure 4.43. The percentage increase in haemoglobin was higher in both the control and the anaemic rats that consumed vitamin A+iron fortified milk lyophilates and those which consumed iron fortified milk lyophilates compared to rats fed with unfortified milk lyophilates. However, % increase in haemoglobin was higher in rats fed with vitamin A+iron fortified milk lyophilates compared to rats fed with iron fortified milk lyophilates suggesting that vitamin A must have played some role in haemoglobin formation. The provision of vitamin A to iron deficient rats resulted in a rapid rise in haemoglobin; leading to a conclusion by Koessler *et al*. (1926) that blood regeneration can not take place without the presence of vitamin A.

Our studies were in agreement with Alferez *et al*. (2006) who also reported that after feeding the iron deficient rats for 14 days with the experimental diets, there was recovery of anaemia, as assessed by higher Hb level. Our haematological data were in agreement with data from other studies (Pallare´s *et al*. 1993; Wienk *et al.* 1996). Our results were in corroboration with Alferez *et al*. 2006; Benito *et al*. 1998 and Pallares *et al*. 1993 who also reported greater digestive utilisation of iron by anaemic rats compared to control rats when they were fed with the experimental diets.

Hunter (1978) also found that haemoglobin concentration depleted continually during the first 4-5 weeks of feeding the rats with iron deficient diets. When rats fed with iron deficient diets were switched to the control levels, their haemoglobin levels increased to control levels within first week. There was an increase in the haemoglobin content after rats were fed with fruit juice fortified with different iron salts for 21 days (Haro-Vicente *et al.* 2008).

4.18.2.5 Plasma ferritin content of rats

Ferritin is a water soluble iron storage protein. Ferritin levels are an indicator of the amount of iron absorbed by the body. The ferritin level in the rat plasma samples was measured using a highly sensitive two site enzyme linked immunosorbent assay (ELISA). Blood was collected in vaccutainers internally coated with heparin and centrifuged at 1500 rcf for 15 minutes at 4ºC to separate plasma as the supernatant. Plasma was collected in another tube and kept at -20ºC.

4.18.2.5.1 Plasma ferritin content in control group rats

Plasma ferritin content of control group rats was 1661.72 ng/ml and increased to 2112.89, 2187.58 and 2368.83 ng/ml for control A group, control B group and control C group, respectively as shown in table 4.107. Percentage increase in ferritin content after 21 days feeding was 27.15, 31.65 and 42.55% for control A group, control B group and control C group, respectively. There was a significant difference in ferritin content and % increase in ferritin content of all the groups ($P<0.05$), being highest for control C group i.e. vitamin A+iron group suggesting that vitamin A improved the utilisation of iron and its incorporation to form ferritin.

Table 4.107: Plasma ferritin content in control group rats

Group	Ferritin content 0 day (ng/ml plasma)	Ferritin content after 21 days (ng/ml plasma)	% ↑ in ferritin content from 0 day
Control A	1661.72±173.83	2112.89±37.63^{a}	27.15±2.27^{a}
Control B	1661.72±173.83	2187.58±33.00^{b}	31.65±2.06^{b}
Control C	1661.72±173.83	2368.83±35.95^{c}	42.55±2.16^{c}

Data are presented as means±SEM (n=8).
$^{a-b}$Means with same superscript in a column do not vary significantly ($P<0.05$) from each other.

4.18.2.5.2 Plasma ferritin content of anaemic group rats

Plasma ferritin content of anaemic rats was 1604.34 ng/ml at 0 day of pre-experimental period, which decreased to 1304.10 ng/ml after feeding the rats with low iron/anaemic diet for 21 days. Ferritin content of anaemic rats increased to 1675.24, 2006.17 and 2330.78 ng/ml for anaemic A group, anaemic B group and anaemic C group, respectively after feeding with their respective diets for 21 days as shown in table 4.108. The tendency of plasma ferritin concentration to decrease in rats during iron deficiency was consistent with the observations of Jacobs *et al.* (1972); Lipschitz *et al.* (1974); Siimes *et al*. (1974) and Jacobs *et al*. 1975 that humans with iron deficiency anaemia have low serum ferritin levels.

Percentage increase in ferritin content after 21 days feeding was 28.46±2.87, 52.82±8.82 and 79.14±4.88% for anaemic A group, anaemic B group and anaemic C group, respectively. There was a significant difference in ferritin content and % increase in ferritin content of all the groups ($P<0.05$), being highest for anaemic C group i.e. vitamin A+iron group suggesting that vitamin A inclusion in diet helped in increasing absorption of iron, thereby increasing ferritin level of blood plasma. In the rats fed with fortified diets, there was significant difference in % increase in ferritin content in control and anaemic group rats ($P<0.05$).

Table 4.108: Plasma ferritin content in anaemic group rats

Group	Ferritin content 0 day PEP (ng/ml plasma)	Ferritin content 0 day EP (ng/ml plasma)	Ferritin content 21 days (ng/ml plasma)	% ↑ in ferritin content from 0 day of EP
Anaemic A	1604.34±33.12	1304.10±38.84	1675.24±37.44^{a}	28.46±2.87^{a}
Anaemic B	1604.34±33.12	1304.10±38.84	2006.17±114.63^{b}	52.82±8.82^{b}
Anaemic C	1604.34±33.12	1304.10±38.84	2330.78±68.65^{c}	79.14±4.88^{c}

Data are presented as means±SEM (n=8).
$^{a\text{-}b}$Means with same superscript in a column do not vary significantly (P<0.05) from each other.
PEP=pre-experimental period
EP= experimental period

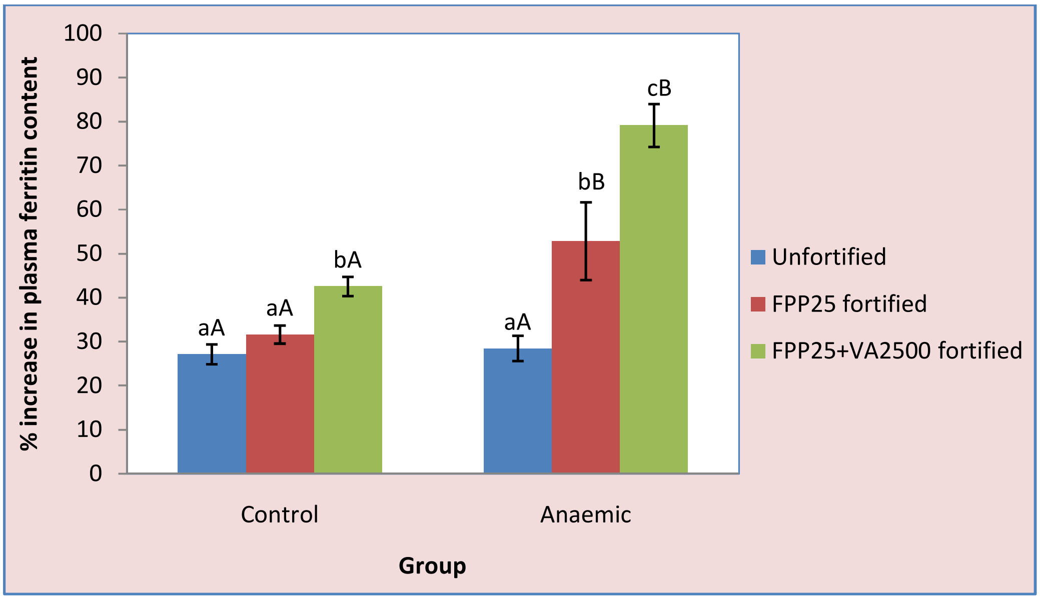

Figure 4.44: Percent increase in plasma ferritin content in control and anaemic group rats.

$^{a\text{-}b}$Different lowercase letters denote significant difference (P<0.05) across sub-groups (unfortified, FPP25 fortified and VA2500+FPP25 fortified).
$^{A\text{-}B}$Different uppercase letters denote significant difference (P<0.05) between groups (control and anaemic).
Error bars show the variations of eight determinations in terms of standard error of mean.

Two way ANOVA (P<0.05) showed that increase in ferritin content was higher for anaemic rats than control group and the difference was statistically significant suggesting that low iron status of anaemic rats lead to increase in uptake of iron and thereby enhancing ferritin synthesis more than the rats having normal iron status (figure 4.44).

Our results were in accordance with Zhu *et al*. (2009) who studied the effect of food matrix (non fat dry milk powder) on iron bioavailability in combined *in vitro* digestion and caco-2 cell model. They compared the bioavailability of soluble ferric pyrophosphate with that of monosodium ferric EDTA and ferrous sulphate. They further reported that ferritin formation in caco-2 cells exposed to soluble ferric pyrophosphate was twice the amount of that in cells treated with ferrous sulphate. They also found that iron free non fat dry milk powder alone was shown to be a poor source of bioavailable iron. Adding soluble ferric pyrophosphate or ferrous sulphate significantly increased ferritin formation, and the magnitude of increase was similar. Ferritin formation from caco-2 calls treated with monosodium ferric EDTA enriched non fat dry milk powder was also significantly higher than the non fat dry milk powder alone, however, the magnitude of increase was only about 60% of the soluble ferric pyrophosphate-enriched ones.

Hunter (1978) concluded that serum ferritin concentration is not a reliable measure of iron status in rats. The author reported that due to animal-to-animal variability in the plasma (serum) ferritin concentration, it is uncertain how rapidly plasma concentration is depleted during iron deficiency. During iron repletion, the plasma ferritin data were so variable that no conclusion could be drawn about the response of plasma ferritin concentration to dietary iron. Similarly, Ward *et al*. (1977) observed non significant difference in serum ferritin levels in rats fed an iron-deficient diet compared to rats fed iron-supplemented diets.

Hunter (1978) reported that liver and spleen ferritin concentrations decreased in parallel during iron depletion and increased in parallel during iron repletion and suggested that liver and spleen ferritin are better indicators of iron status than plasma ferritin.

However, in the present investigation, plasma ferritin responded according to the iron status and proved to be a good indicator of iron depletion and repletion. Ferritin content of the rats fed with vitamin A+iron fortified milk lyophilates was higher compared to other two groups suggesting complementary role of vitamin A in iron absorption. However, Mejia and Chew (1988) reported that vitamin A supplementation produced non significant change in serum ferritin. Siimes *et al.*

(1974) found a rapid rise in serum ferritin concentration in the first week of iron treatment of anaemic children.

4.18.2.6 Plasma transferrin content of rats

Transferrin is iron-binding blood plasma glycoprotein that controls the level of free iron in biological fluids. It has the capacity to pick up iron from tissues throughout the body. Each transferrin molecule can carry multiple atoms of iron much like a bus can carry multiple passengers. The transferrin level in the rat plasma samples was measured using a highly sensitive two site enzyme linked immunosorbent assay (ELISA). Blood was collected in vaccutainers internally coated with heparin and centrifuged at 1500 rcf for 15 minutes at 4ºC to separate plasma as the supernatant. Plasma was collected in another tube and kept at -20ºC.

4.18.2.6.1 Plasma transferrin content of control group rats

Plasma transferrin content of control group rats was 1595.62 µg/ml and it increased to 1812.96, 2096.60 and 2254.85 µg/ml for control A group, control B group and control C group, respectively after feeding with their respective diets for 21 days as shown in table 4.109. Percentage increase in transferrin content after 21 days feeding was 13.62, 31.40 and 41.32% for control A group, control B group and control C group, respectively. There was significant difference in transferrin content and % increase in transferrin content of all the groups ($P<0.05$), being highest for control C group i.e. vitamin A+iron group suggesting that vitamin A increased iron uptake thereby increasing transferrin content of blood plasma.

Table 4.109: Plasma transferrin content in control group rats

Group	Transferrin content 0 day (µg/ml plasma)	Transferrin content after 21 days (µg/ml plasma)	% ↑ in transferrin content from 0 day
Control A	1595.62±56.83	1812.96 ± 18.48^{a}	13.62 ± 1.16^{a}
Control B	1595.62±56.83	2096.60 ± 17.00^{b}	31.40 ± 1.07^{b}
Control C	1595.62±56.83	2254.85 ± 25.89^{c}	41.32 ± 1.62^{c}

Data are presented as means±SEM (n=8).
$^{a-b}$Means with same superscript in a column do not vary significantly ($P<0.05$) from each other.

4.18.2.6.2 Plasma transferrin content of anaemic group rats

Plasma transferrin content of anaemic rats was 1601.02 µg/ml at 0 day of pre-experimental period, which decreased to 1415.42 µg/ml after feeding the rats with low iron/anaemic diet for 21 days. Transferrin content of anaemic rats increased to 1977.02, 2180.71 and 2307.44 for anaemic A group, anaemic B group and anaemic C group, respectively after feeding with their respective diets for 21 days. Percentage increase in transferrin content after 21 days feeding was 35.65, 54.23 and 63.14% for anaemic A group, anaemic B group and anaemic C group, respectively (as shown in table 4.110).

There was a significant difference in transferrin content and % increase in transferrin content of all the groups (P<0.05), being highest for anaemic C group i.e. vitamin A+iron group suggesting that vitamin A might have played role in enhancement of iron absorption, thereby increasing transferrin level of blood plasma. Mejia and Chew (1988) reported that there was a significant elevation in transferrin saturation when rats were fed with diet supplemented with vitamin A.

Table 4.110: Plasma transferrin content in anaemic group rats

Group	Transferrin content PEP 0 day (µg/ml plasma)	Transferrin content 0 day EP (µg/ml plasma)	Transferrin content 21 days (µg/ml plasma)	% ↑ in transferrin content from 0 day of EP
Anaemic A	1601.02±27.10	1415.42±16.94	1977.02±77.89^{a}	35.65±5.81^{a}
Anaemic B	1601.02±27.10	1415.42±16.94	2180.71±16.96^{b}	54.23±2.23^{b}
Anaemic C	1601.02±27.10	1415.42±16.94	2307.44±24.25^{c}	63.14±2.15^{c}

Data are presented as means±SEM (n=8).
$^{a-b}$Means with same superscript in a column do not vary significantly (P<0.05) from each other.
PEP=pre-experimental period
EP=experimental period

Two way ANOVA (P<0.05) revealed that percent increase in plasma transferrin content was higher for anaemic group compared to control group and significantly different for all the three diets and the values were significantly different from each other in the respective subgroups (figure 4.45).

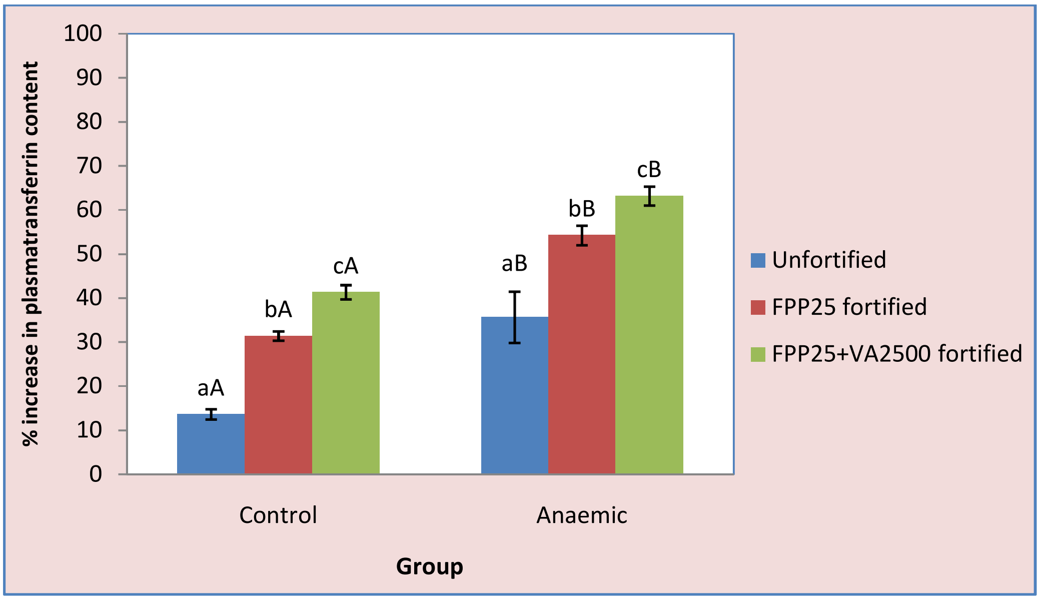

Figure 4.45: Percent increase in plasma transferrin content in control and anaemic group rats

$^{a\text{-}b}$Different lowercase letters denote significant difference (P<0.05) across sub-groups (unfortified, FPP25 fortified and VA2500+FPP25 fortified).

$^{A\text{-}B}$Different uppercase letters denote significant difference (P<0.05) between groups (control and anaemic).

Error bars show the variations of eight determinations in terms of standard error of mean.

Conceicao *et al*. (2001) reported that iron supplementation (Fe^{2+} or Fe^{3+}) normalised the serum transferrin levels in iron-deficient rats more than the control group. Stuijvenberg *et al.* (2006) observed significant increase in transferrin saturation in children given brown bread fortified with ferrous bisglycinate. Haro-Vicente *et al*. (2009) reported that transferrin saturation was significantly (*P*<0.05) higher in groups (infants and toddlers) fed with iron-fortified growing-up milk compared to the control group.

Tissues that have a high uptake of e.g. erythroid precursors, liver and placenta possess large number of transferrin receptors, which have been shown to change in response to changes in iron available to or needed by the cell i.e. the removal of iron from circulation would be controlled by the number of transferrin receptors on body cells (Benitto and Miller 1998).

4.18.2.7 Vitamin A status of liver of rats

4.18.2.7.1 Vitamin A and iron status of liver of control group rats

There was slight but non significant difference ($P<0.05$) in vitamin A content of liver of control group A rats and control group B rats, however, vitamin A content of control group C rats was significantly higher than group A and B (3.94, 5.16 and 15.49 µg/g liver for group A, group B and group C, respectively) (table 4.111). Our results were in accordance with Burri and Jacob (1988) who fed the rats with diets containing different amounts of retinyl palmitate (RP) and found that retinyl palmitate stored in the liver of these rats differed with the different vitamin A supplemented diets and was substantially (400%) greater in rats fed 10.0 mg RP/kg than in rats fed 0.5 RP mg/kg diet.

Rosales *et al.* (1999) reported that liver vitamin A concentration was higher in the group receiving 35 mg iron with more hepatic accumulation of retinol and retinyl etser compared to other groups. On the other hand, all the control groups differed significantly from each other in terms of iron status of liver (141.54, 174.43 and 232.22 ppm for group A, group B and group C, respectively). Similarly, Dabbagh *et al.* (1994) reported that iron content in the liver of rats supplied with excess amount of iron was 1391 µg/g liver compared to 103.9 µg/g in case of control group rats. Alferez *et al*. (2006) reported that iron content of liver was higher for the rats fed with goat milk diet (having high vitamin A content than other diets studied).

Table 4.111: Vitamin A and iron status of liver of control group rats

Group	Vitamin A content (µg/g liver)	Iron content in liver (ppm)
Control A	3.95 ± 0.45^{a}	141.54 ± 12.10^{a}
Control B	5.16 ± 0.40^{a}	174.43 ± 16.84^{b}
Control C	15.49 ± 1.26^{b}	232.22 ± 13.59^{c}

Data are presented as means±SEM (n=8).
$^{a-b}$Means with same superscript in a column do not vary significantly ($P<0.05$) from each other.

4.18.2.7.2 Vitamin A and iron status of liver of anaemic group rats

All the anaemic groups differed significantly from each other in terms of vitamin A status of liver (3.42, 6.99 and 18.03 µg/g liver for group A, group B and group C, respectively) ($P<0.05$). Similarly, there was a significant difference in iron status of liver in all anaemic groups (146.86, 185.78 and 244.48 ppm for group A, group B and group C, respectively) as shown in table 4.112.

Table 4.112: Vitamin A and iron status of liver of anaemic group rats

Group	Vitamin A Content (µg/g liver)	Iron content in liver (ppm)
Anaemic A	3.42±0.39^{a}	146.86±7.21^{a}
Anaemic B	6.99±0.33^{b}	185.78±19.28^{b}
Anaemic C	18.03±0.54^{c}	244.48±10.99^{c}

Data are presented as means±SEM (n=8).
$^{a-b}$Means with same superscript in a column do not vary significantly (P<0.05) from each other.

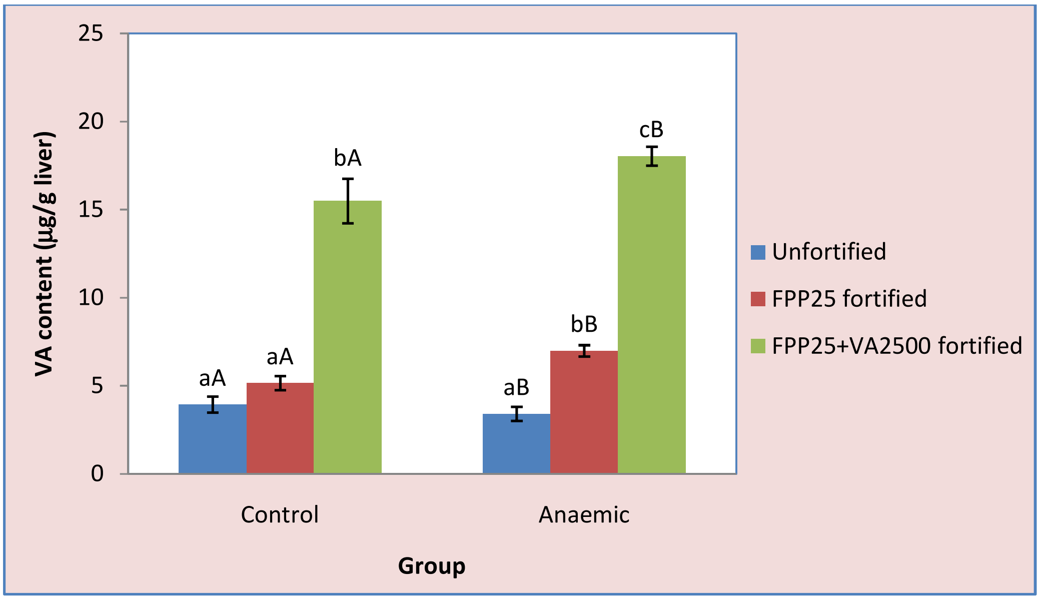

Figure 4.46: Vitamin A content of liver of control and anaemic group rats
$^{a-b}$Different lowercase letters denote significant difference (P<0.05) across sub-groups (unfortified, FPP25 fortified and VA2500+FPP25 fortified).
$^{A-B}$Different uppercase letters denote significant difference (P<0.05) between groups (control and anaemic).
Error bars show the variations of eight determinations in terms of standard error of mean.

Two way ANOVA (P<0.05) revealed that for all the three diets, there was significant difference (P<0.05) in vitamin A content in liver of control and anaemic rats (figure 4.46). However, it can be inferred from the results of present investigation that during 21 days of feeding of rats with low iron diet, vitamin A stores of liver might not have been depleted to the extent that they would have to take very long time to get repleted. Therefore, when the rats receiving low iron diets (partially depleted liver vitamin A stores) were switched to normal diets, they responded more for vitamin A storage in liver compared to control rats whose vitamin A stores were absolutely normal. Increased status of vitamin A of liver of anaemic rats could be ascribed to the enhanced absorption of vitamin A caused by anaemia as shown in balance studies for vitamin A; and storage of extra amount of vitamin A supplied to rats other than the actual requirement.

Our results were in accordance with Wieringa *et al.* (2007) who reported that liver vitamin A store was greater in the vitamin A deficient rats which were supplemented with iron for 4-6 months. They suggested a possible redistribution of serum retinol and thus impairment in vitamin A deficiency observed after supplementation. A significant increase was observed in serum retinol concentration when women were given diets fortified with vitamin A and iron.

Studies on rats have shown that iron deficiency is associated with low plasma retinol concentration and increased hepatic vitamin A, a higher molar ratio of hepatic retinyl ester to retinol (increased vitamin A deposition), suggesting that liver vitamin A metabolism is altered and the mobilisation of this vitamin might be impaired in the presence of iron deficiency. These changes might result from a reduced hepatic retinyl ester or from an increased esterification of hepatic retinol (Rosales *et al.* 1999; Jang *et al.* 2000; Strube *et al.* 2002).

Jang *et al.* (2000) found that after 15 weeks of dietary treatment, plasma retinol concentration in rats receiving the low iron diet was significantly lower than in rats receiving the control diet (1.34±0.11 vs 0.53±0.11 mmol/L, $P<0.001$). In contrast, liver vitamin A status in rats receiving reduced iron was greater than in rats receiving the control diet (808.5±94.0 vs 112.2±23.5 nmol/L, $P<0.05$), thus indicating that this situation might have resulted from a reduction in the activity of one or more retinyl ester hydrolases, with a consequent decrease in vitamin A mobilisation even in the presence of sufficient or increased quantities of this vitamin.

Oliveira *et al*. (2008) showed that iron deficiency seems to deteriorate vitamin A metabolism leading to a reduction in serum retinol and an increase in hepatic retinol and retinyl ester. These alterations probably result from an increase in retinol sequestration to the liver and/or impairment in the activity of hepatic retinyl ester hydrolases decreasing vitamin A mobilisation.

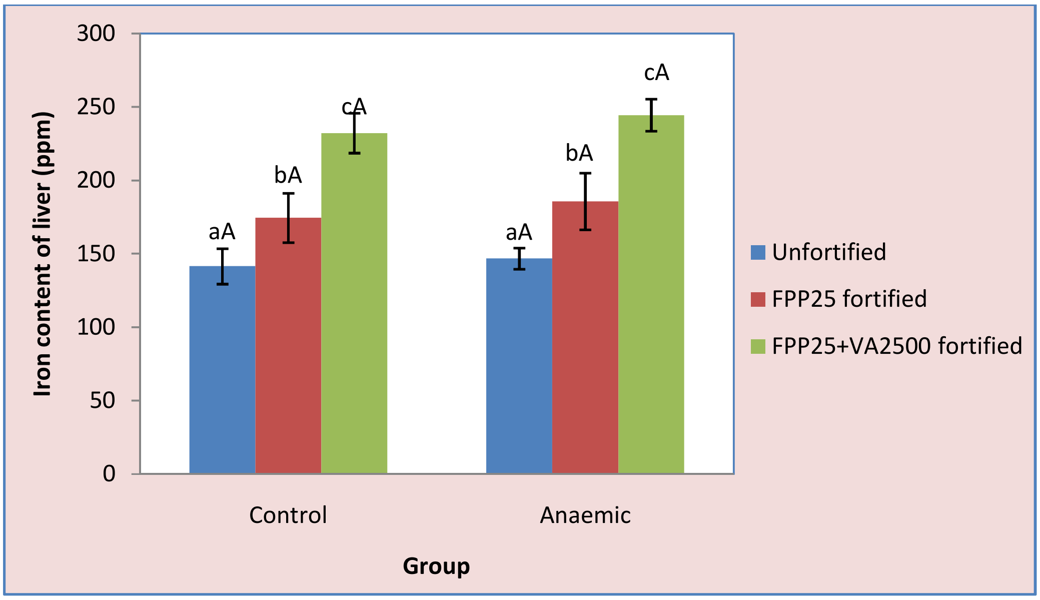

Figure 4.47: Iron content of liver of control and anaemic group rats
$^{a-b}$Different lowercase letters denote significant difference (P<0.05) across sub-groups (unfortified, FPP25 fortified and VA2500+FPP25 fortified).
$^{A-B}$Different uppercase letters denote significant difference (P<0.05) between groups (control and anaemic).
Error bars show the variations of eight determinations in terms of standard error of mean.

Two way ANOVA (P<0.05) revealed that for all the diets studied, there was non significant difference (P<0.05) in iron status of control and anaemic rats (figure 4.47). Our results were in accordance with Haro-Vicente *et al*. (2008) who reported that the iron content in the liver of iron deficient rats fed with ferrous sulphate and micronised dispersible ferric pyrophosphate fortified fruit juice for 21 days was significantly higher than control group. However, Alferez *et al.* (2006) reported that iron concentrations decreased in all the tissues of anaemic rats that were analysed, compared with their controls, regardless of the diet supplied. They found that both the diet and the anaemia had a significant effect on iron deposits in liver. Similar findings have been reported by Katsuhiko *et al*. (1991). They concluded that the marked decrease in iron concentration in the liver is suggestive of the depletion of iron storage in anaemic rats.

It is usually accepted that the sequence of events during oral treatment of iron-deficiency anaemia is, first, the normalisation of the haemoglobin value, followed by restoration of storage iron (Bothwell and Finch, 1962). Under the experimental conditions used by Alferez *et al*. (2006), there was an inverse correlation between haemoglobin-iron gain and liver stores. This means that although anaemic rats responded rapidly to iron dietary repletion with respect to haematology and storage of non-haeme organ iron, the anaemic rats showed a significantly lower iron concentration than did their controls. They concluded that there was an incomplete restoration of iron stores within the time frame (14 days) of their experiment. Their results were consistent with the frequently cited assumption that iron stores are lost more rapidly than haemoglobin iron during iron deficiency and are restored more slowly than haemoglobin iron during iron repletion (Moore 1973).

Hunter (1978) reported that it would seem reasonable to expect that anaemic animals would need to restore their oxidative metabolism (by increasing haemoglobin synthesis) before they would need to replenish their iron stores. Changes in total liver iron during iron depletion and repletion roughly paralleled the changes in liver ferritin concentration.

However, in the present study, higher iron content of livers of anaemic rats could be due the longer repletion time (21 days) as compared to 14 days repletion time in study conducted by Alferez *et al.* (2006).

On the same line as in case of control group, iron status of liver was higher for rats receiving diets fortified with vitamin A+iron. The findings were in support with Alferez *et al.* (2006) who reported that iron content of liver was higher for the rats fed with goat milk diet (having high vitamin A content than other diets studied).

4.18.2.8 Effect of vitamin A and iron fortification on digestibility of milk proteins

There was non significant difference in protein quality parameters for both control and fortified group ($P<0.05$) as shown in figure 4.48. Digestibility coefficient of control group was slightly more than fortified group, however the values did not differ significantly (65.50 and 64.35% for control and fortified group, respectively).

On the other hand, biological value and net protein utilisation of fortified group were slightly higher than the control group, however, statistically, the difference was non significant (biological value was 80.03 and 82.99% for control and fortified group, respectively; net protein utilisation was 52.39% and 53.40% for control and fortified group, respectively) as shown in table 4.113. The results indicated that there was non significant effect of vitamin A+iron fortification on protein digestibility of milk proteins.

Table 4.113: Effect of vitamin A and iron fortification on milk protein quality parameters

Group	Digestibility coefficient (%)	Biological value	Net protein utilisation (NPU)
Control (unfortified)	65.50±1.31^{a}	80.03±2.30^{a}	52.39±1.70^{a}
VA+FPP Fortified	64.35±1.10^{a}	82.99±0.77^{a}	53.40±1.00^{a}

Data are presented as means±SEM (n=8).
$^{a-b}$Means with same superscript in a column do not vary significantly (P<0.05) from each other.

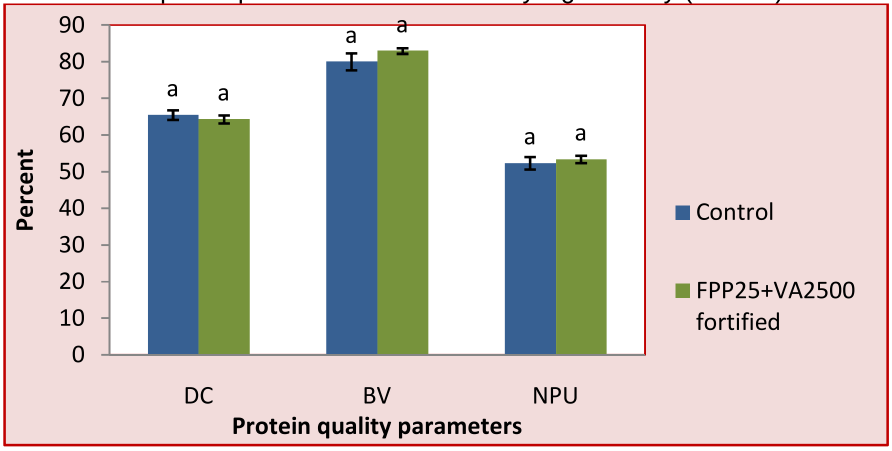

Figure 4.48: Effect of vitamin A and iron fortification on digestibility coefficient (DC), biological value (BV) and net protein utilisation (NPU) of milk proteins.

$^{a-b}$Samples represented with different letters are significantly different (P<0.05) from each other.
Error bars show the variations of eight determinations in terms of standard error of mean.

In nutshell, the results of animal study revealed that under our experimental conditions, diet fortified with both vitamin A and iron resulted in better bioavailability of iron and vitamin A than diet fortified with iron or vitamin A alone. Multiple fortification of milk increased the digestive and metabolic utilisation and deposition of iron and vitamin A in target organs and favoured the recovery of normal haematological parameters after nutritional ferropenic anaemia.

Our results were in accordance with Ranhotra *et al.* (1981) and Zhu *et al.* (2009). Ranhotra *et al.* (1981) used a water soluble citrate phosphate iron complex (iron 16.67%) to fortify milk (38 ppm iron) and showed that iron in milk was as well available as that in ferrous sulphate (99% vs 100%); the same was true when iron complex was tested as such (not added to milk). Thus, milk or milk components appeared not to adversely affect the bioavailability of added iron under the conditions used in their experiment.

Zhu *et al*. (2009) also reported that soluble ferric pyrophosphate exhibited higher bioavailability than simple iron salts, such as ferrous sulphate and ferric chloride. The bioavailability of soluble ferric pyrophosphate may be related to its iron core shielded by the surrounding pyrophosphate and citrate ligands which may provide iron as a relatively low molecular weight entity that readily interacts with biological iron receptors, because polymeric iron compounds have significantly slower iron transfer kinetics. They indicated that the ligands do not present a physical barrier or chelate iron with sufficiently high affinity to inhibit iron uptake by the cells and suggested that soluble ferric pyrophosphate is a promising agent for use in food fortification and especially in dairy or lipid based fortification.

Our results were also in consonance with Sun *et al*. (2010) who found an additive effect of retinol and iron supplementation on iron status**.** Retinol plays an important role in improving iron status, enhancing iron utilisation by stimulating erythropoiesis and iron metabolism by raising mean red cell volume and plasma iron concentration (Semba *et al*. 2001). Semba and Bloem (2002) also reported that vitamin A appeared to be involved in the pathogenesis of anaemia through diverse biological mechanisms, such as the enhancement of growth and differentiation of erythrocyte progenitor cells, potentiation of immunity to infection and reduction of the anaemia of infection, and mobilisation of iron stores from tissues.

From the present study, it can be concluded that fortification of milk with ferric pyrophosphate soluble and ferric pyrophosphate soluble+vitamin A acetate had a significant effect on the digestive and metabolic utilisation of the iron as well as on the haematological parameters. All the positive effects were more evident in anaemic rats, indicating the dependence of iron bioavailability on the iron status of individual. Similarly, the increase was greater for vitamin A acetate+ferric pyrophosphate soluble fortified group compared to that with only ferric pyrophosphate soluble, suggesting that vitamin A plays role in enhancement of iron absorption. Normal rats also showed an increase haemoglobin content, plasma ferritin content, plasma transferrin content, percentage digestibility and retention of iron which clearly states that selected salt has a good bioavailability.

CHAPTER –5

Summary and Conclusion

5. Summary and Conclusion

Iron deficiency has earned distinction as the most common nutritional deficiency in the world today. Iron is an essential micronutrient as it plays a vital role in many metabolic processes including oxygen transport, oxidative metabolism, cellular proliferation and growth. Iron balance is rigorously controlled by the regulation of absorption and its deficiency often leads to anaemia, defined as having a blood haemoglobin level below standard which is usually the result of insufficient dietary intake of iron, poor utilisation of iron from ingested food, or a combination of the two. Vitamin A deficiency (VAD) exists in more than 60 countries, at a clinical and/or subclinical level. It is an essential nutrient that is required in small amounts by humans for the normal functioning of the visual system, the maintenance of cell function for growth, epithelial cellular integrity, immune function and reproduction. WHO has defined VAD as tissue concentrations of vitamin A low enough to have adverse health consequences, even if there is no evidence of clinical xerophthalmia.

Food fortification is the most cost effective, sustainable and optimal approach in the battle against micronutrient deficiencies. Milk was selected as the fortification vehicle owing to the fact that it is centrally processed, widely distributed and regularly consumed by all sections of the society in predictable amounts and bioavailability of the added nutrients remains high. Iron fortification of milk and dairy products is also considered as potential approach to prevent iron deficiency disorders. However, results concerning the biophysicochemical modifications, organoleptic changes, iron bioavailability and iron distribution in tissue are conflicting. Indeed, all these modifications depend on different factors, such as the physico-chemistry of the added iron (iron valence, solubility, degree of chelation and complex formation), the physico-chemistry of iron after processing or storage, presence of other components in the meal that can increase or decrease the absorption of iron and the physiological state of the added iron. The main findings of the present investigation have been listed as follows:

1. Sensory analysis, pH, acidity and TBA value tests were conducted to select a suitable iron salt and vitamin A ester for fortification. Milk was

fortified with nine different iron salts and two vitamin A esters; finally, ferric pyrophosphate (FPP) soluble, ferrous gluconate (FG) hydrate (@15, 20 and 25 ppm) and vitamin A acetate (@2500 IU/L) were selected for fortification.

2. Ferrous sulphate microcapsules were prepared by spray cooling method (using sodium alginate/sodium alginate and pectin/polyglycerol monostearate as wall material). There was problem of sedimentation at the bottom with alginate capsules and PGMS capsules floated on the surface and did not mix properly. Ferrous sulphate liposomes were made using liquid soya lecithin, powdered soya lecithin and egg lecithin. The former two were rejected owing to their pronounced odour and poor solubility in milk. Liposomes of ferrous sulphate were prepared using egg lecithin as wall material; flavoured milk (fortified@5, 10 and 15 ppm iron) was prepared using liposomes. Sensory acceptability of fortified milk (@5 ppm) was comparable to control. However, higher level of fortification led to pronounced flavour defects in milk and cost of fortification also increased substantially by additional ingredients used in liposome formation (wall material, solvents and stabilisers), hence, liposomes were not considered for further experiments.
3. Heat stability experiments were conducted for the fortified milk; and it was found to be comparable with control milk in terms of heat coagulation time.
4. Optimum level of iron fortification was found to be 25 ppm and that of vitamin A was 2500 IU/L on the basis of sensory scores and heat stability.
5. Stability of the added nutrients (vitamin A and iron) was evaluated under different processing conditions viz. heat treatments (pasteurisation, boiling and sterilisation), light exposure (1485, 2970 and 4455 lux), packaging materials (polyethylene pouches and glass bottles) and it was found that:
    - 5.1. Added vitamin A (microencapsulated form) was more stable compared to the native vitamin A present in milk due to the shielding effect of the wall material on core material.
    - 5.2. There was a greater degradation of vitamin A in iron fortified milk suggesting that iron might have played a role in accelerating the degradation of vitamin A.

    5.3. FG hydrate caused greater losses of vitamin A compared to FPP soluble, owing to the fact that FPP soluble is a chelated ferric salt (lesser interaction with matrix i.e. milk and its constituents).
    5.4. There was no effect of processing conditions used on the stability of iron in milk.

6. Sensory acceptability and storage stability in terms of pH, acidity, TBA value and oxidative stability of the iron+vitamin A fortified milk were estimated for a period of 7 days; at fixed intervals i.e. 0, 3rd, 5th and 7th day. Iron fortified milk had good sensory acceptability upto 7 days. However, pH, acidity, TBA value and other oxidative stability parameters (peroxide value, % free fatty acids, TBA value and induction period) were negatively affected by fortification. The oxidation of ghee prepared from iron fortified milk was faster as compared to ghee prepared from control milk. The peroxide development was also faster in case of iron fortified samples as compared to control. It was slightly higher in case of FG fortified milk as compared to FPP fortified milk. It is to be pointed out that pH and acidity of milk were not affected drastically on fortification with selected iron salts and vitamin A ester and the magnitudes of changes were small enough to have any detrimental effect on milk system.
7. There was non significant effect of iron and vitamin A fortification on curd tension, viscosity, rennet coagulation time, alcohol stability and colour profile of milk.
8. Dry sediments (%) in fortified milk were slightly higher than that in corresponding control, however, the low values indicated that fortified milk had good heat stability and was suitable for high temperature processing.
9. Distribution study clearly showed that most of the added iron (for both FPP soluble and FG hydrate) was retained in skim milk fraction and the remaining was in fat phase (ghee and ghee residue). Out of the total iron present in skim milk, major portion was bound to casein and the remaining was bound to whey proteins.
10. Ultracentrifugation of milk revealed that 88-90% iron in both control and fortified milk was present in colloidal phase and the remaining was in soluble phase suggesting that the binding sites of casein were not saturated and could bind with more iron ions when added to milk.

11. Electrophoretic pattern of milk proteins (control and fortified milk) as determined by urea-PAGE and SDS-PAGE was not affected by fortification.
12. Maillard browning (HMF content and furosine content) was higher in iron fortified milk compared to control milk. The magnitude of difference in HMF content and furosine content was found to be higher for FG hydrate compared to FPP soluble owing to the chelated nature of the latter salt. HMF and furosine content was significantly higher in boiled and sterilised samples compared to their pasteurised counterparts.
13. *Dahi* and yoghurt prepared from fortified milk had comparable sensory, microbiological and physicochemical properties (pH, acidity, setting time, syneresis, water holding capacity, viscosity and texture profile) with those prepared from control milk.
14. Addition of iron upto 25 ppm did not cause any detrimental effect on milk system as indicated by the changes in sensory acceptability, pH, acidity, TBA value, vitamin A and iron retention and oxidative stability parameters. Therefore, 25 ppm level of fortification was found to be optimum for selected iron salts.
15. Results of *in vitro* trials revealed that % bioaccessibility of iron and vitamin A in fortified milks was comparable to those present in control milk.
16. Magnitude of changes caused in the milk system was higher in case of FG hydrate compared to FPP soluble (pH, acidity, TBA value, vitamin A losses, oxidative stability parameters, HMF content, furosine content and other physico-chemical parameters). Therefore, FPP soluble@25 ppm and vitamin A acetate@2500 IU/L were selected for conducting *in vivo* study.
17. *In vivo* trials were conducted in rats to validate the anti-anaemic properties of the iron+vitamin A fortified milk and effect of vitamin A on enhancement of iron status and vice versa. Biological indices (apparent digestibility coefficient, iron balance and retention/intake) were also estimated to find out the metabolic and digestive utilisation of iron and vitamin A; effect of vitamin A on iron absorption and vice versa. Iron and vitamin A status of rat liver were also estimated. The present study made it evident that:
    - 17.1. Iron+vitamin A fortification of milk with FPP soluble had significant effect on the digestive and metabolic utilisation of iron and vitamin A

as well as on the haematological parameters (haemoglobin, plasma ferritin and plasma transferrin).

17.2. Vitamin A enhanced iron absorption and vice-versa; as suggested by balance studies, haematological parameters and iron and vitamin A status of liver.

17.3. Positive effects of fortification were more evident in anaemic rats which showed the dependence of iron and vitamin A bioavailability on the iron status of the individual.

17.4. Normal rats also showed an increased percentage digestibility and retention of vitamin A and iron, liver status of iron and vitamin A, Hb value, plasma ferritin concentration and plasma transferrin concentration which clearly indicated that the selected fortificants had good bioavailability.

17.5. Protein quality parameters (digestibility coefficient, biological value and net protein utilisation) were not affected by iron+vitamin A fortification suggesting that these added micronutrients had no adverse effect on protein digestibility.

The current study led to the development of iron+vitamin A fortified toned milk. A 250 ml serving of the fortified milk will provide ~30-35% of iron and vitamin A requirement on daily basis. An additional cost of 36 paise (cost of added nutrients i.e. vitamin A acetate and FPP soluble) will be incurred over the normal processing cost in the formulation of one litre of fortified milk (Annexure III). The product had high heat stability and good sensory acceptability upto 7 days storage. The biological efficacy of fortifying milk with vitamin A and iron was validated through *in vitro* and *in vivo* experiments. Fortified foods are of unquestionable value in protecting the nutritional status of vulnerable groups and people affected by emergencies. This multiple fortified milk will have the dual advantage of being able to deliver both vitamin A and iron to large segments of the population without requiring radical changes in food consumption patterns.

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Annexures

Annexure I

SENSORY CARD FOR MILK

Date: **Storage time:**

Name of panelist :

Objective : To evaluate vitamin A and iron fortified milk for its sensory attributes

A) Assign score for each sample for different characteristics

Characteristics	Maximum score	Sample score 1	2	3	4	5
1. Colour and appearance	10					
2. Odour	20					
3. Taste	40					
4. Mouthfeel	30					

B) Indicate the degree of defects such as the following. Encircle the one applicable and deduct from attributes.

Characteristics	Defect	Degree of defect Suspicious	Slight	Pronounced
1. Colour and appearance	Off-colour, suspended particles, filth, foreign matter, reddish tinge	2	4	10
2. Odour	Stale, acidic, abnormal, rancid	5	10	15
3. Taste	Oxidised, rancid, metallic	5	10	20
4. Mouthfeel	Watery, heavy, curdy, chalky	5	10	15

Comments:

Signature

Annexure II

SCORE CARD FOR *DAHI*/YOGHURT

Date: **Storage time:**

Name of panelist :
Objective : To evaluate *dahi*/yoghurt prepared from control and fortified milk for sensory attributes

A) Assign score for each sample for different characteristics

Attribute	**Maximum score**	**Sample score**		
		1	**2**	**3**
Flavour	45			
Body & texture	30			
Acidity	10			
Colour and appearance	10			
Container and closure	5			
Total	100			

B) Indicate the degree of defects such as the following. Encircle the one applicable and deduct from attributes.

Attribute	Defect	Intensity of defect		
		Slight	Definite	Pronounced
Flavour	Highly acidic, bitter, metallic,	7	9	11
	yeasty, cheesy	10	13	16
Body and texture	Grainy, thin body	2	5	8
	ropy and wheying off	4	8	12
Acidity	Too low, too high	1	3	5
Colour and appearance	Unnatural colour,	1	3	5
	presence of foreign matter	2	4	6
Container and closure	Soiled, improperly covered	1	2	3

Comments:

Signature

Annexure III

COST CALCULATION FOR VITAMIN A AND IRON FORTIFICATION OF MILK

Level of vitamin A required for fortification = 2500 IU/L

Potency of vitamin A acetate 250 CWS = 250000 IU/g or 250000/1000 mg

Quantity required for vitamin A fortification of 1 L milk@2500 IU/L = $\frac{1000 \times 2500}{250000}$

= 10 mg

Cost of 50 g vitamin A acetate 250 CWS (Piramal Healthcare Ltd., Mumbai, India) = 100 Rs. or 10000 Paise

Cost of 10 mg vitamin A acetate 250 CWS = $\frac{10000 \times 10}{50 \times 1000}$

= 2 paise

Cost of fortification of milk with vitamin A acetate@2500 IU/L = 2 paise

Level of iron required for fortification = 25 ppm

Iron content of ferric pyrophosphate (FPP) soluble = 13.12%

Quantity of FPP soluble required to fortify 1 L milk@25 ppm iron = 198.52 mg

Cost of 250 g FPP soluble (Shanpar Industries Pvt. Ltd., Vadodara, Gujarat, India) = 425 Rs. or 42500 paise

Cost of 198.52 mg FPP soluble = $\frac{42500 \times 198.52}{250 \times 1000}$

= 33.75 paise

= 34 paise

Cost of fortification of milk with FPP soluble@25 ppm iron = 34 paise

Cost of fortification of 1 L milk with vitamin A acetate@2500 IU/L+ 25 ppm iron as FPP soluble = 2 + 34 = 36 paise