# DEVELOPMENT AND EVALUATION OF MILK PROTEIN-VITAMIN A COMPLEXES

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

**DOCTOR OF PHILOSOPHY**

**IN**

**DAIRY CHEMISTRY**

**BY**


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KARNAL-132001 (HARYANA), INDIA


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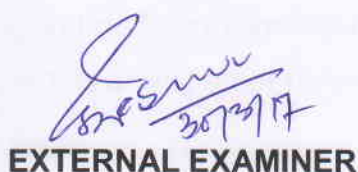

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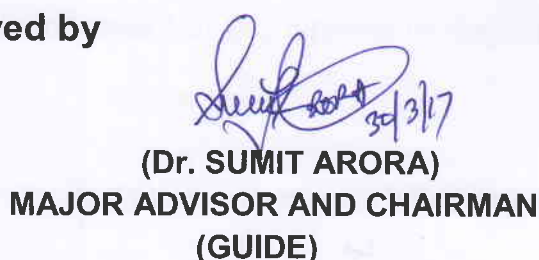

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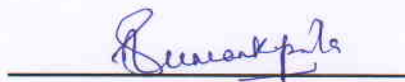

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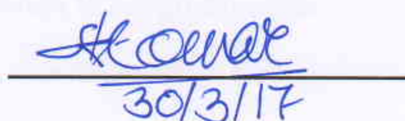

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This is to certify that the thesis entitled "**DEVELOPMENT AND EVALUATION OF MILK PROTEIN-VITAMIN A COMPLEXES**" submitted by **Ms. CHITRA GUPTA** in partial fulfillment of the requirement for award of the degree of **DOCTOR OF PHILOSOPHY** in **DAIRY CHEMISTRY** of the **ICAR-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.

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Dedicated

To

Beloved Family

&

Respected Guide

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# List of Abbreviations

| | |
|---|---|
| ANS | 8-anilino-1-naphthalene sulfonic acid |
| Ala | Alanine |
| $NH_3$ | Ammonia |
| $NH_4OH$ | Ammonium Hydroxide |
| APS | Ammonium persulphate |
| Arg | Arginine |
| Asn | Asparagine |
| Asp | Aspartic acid |
| AOAC | Association of Official Analytical Chemists |
| $H_3BO_3$ | Boric acid |
| BSA | Bovine serum albumin |
| $CaCl_2$ | Calcium chloride |
| CNP | Casein nanoparticles |
| Cys | Cystiene |
| $K_2HPO_4$ | Di potassium hydrogen phosphate |
| $CaHPO_4 \cdot 2H_2O$ | Dicalcium phosphate dihydrate |
| DMSO | Dimethyl sulphoxide |
| DHA | Docosahexenoic acid |
| FAO | Food and Agriculture Organisation |
| FDA | Food and Drug Administration |
| FSSAI | Food Safety and Standards Authority of India |
| FSSR | Food Safety and Standards Regulations |
| FTIR | Fourier transform infrared spectroscopy |
| Glu | Glutamic acid |
| Gly | Glycine |
| g | Gram |
| GDP | Gross Domestic Product |
| HCT | Heat coagulation time |
| HDPE | High density polyethylene |
| HPLC | High pressure liquid chromatography |
| His | Histidine |
| h | Hour |
| HCl | Hydrochloric acid |
| Igs | Immunoglobulins |
| ICMR | Indian Council of Medical Research |
| ICN | International Conference on Nutrition |
| IU | International unit |
| IDD | Iodine deficiency disorders |
| IDA | Iron deficiency anaemia |

| Ile | Isoleucine |
|---|---|
| KDa | Kilo Dalton |
| Leu | Leucine |
| L | Litre |
| LDPE | Low density polyethylene |
| Lys | Lysine |
| Met | Methionine |
| µl | Microliter |
| µm | Micrometer |
| mm | Millimeter |
| MPC | Milk protein concentrate |
| mA | Milliampere |
| ml | Millilitre |
| mV | Millivolt |
| min | Minute |
| TEMED | N, N, N’, N’-tetramethylenediamine |
| nm | Nanometre |
| NFHS | National Family Health Survey |
| NHRP | National Health Research Policy |
| NNMB | National Nutrition Monitoring Bureau |
| NNP | National Nutrition Policy |
| NPPNB | National Prophylaxis Programme against Nutritional blindness |
| NSSO | National Sample Survey Office |
| N | Normality |
| NHD | Nutrition for Health & Development |
| ppm | Parts per million |
| Pa | Pascal |
| Phe | Phenylalanine |
| PET | Polyethylene terephthalate |
| KOH | Potassium hydroxide |
| Pro | Proline |
| r-CM | Reassembled casein micelles |
| RNaCas | Reassembled sodium caseinate |
| RSNaCas | Reassembled succinylated sodium caseinate |
| RP-HPLC | Reverse phase–High performance liquid chromatography |
| rpm | Revolutions per minute |
| SEM | Scanning electron microscopy |
| s | Second |
| Ser | Serine |
| NaCas | Sodium caseinate |

| NaCl | Sodium chloride |
|---|---|
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | Sodium dodecyl sulphate |
| NaOH | Sodium hydroxide |
| SNF | Solid not fat |
| SO | Sonication |
| SEM | Standard error mean |
| ST | Stirring |
| SMPC | Succinylated milk protein concentrate |
| SNaCas | Succinylated sodium caseinate |
| SDE | Sustainable Development and Healthy Environments |
| Thr | Threonine |
| TFA | Trifluoro acetic acid |
| Trp | Tryptophan |
| Tyr | Tyrosine |
| UF | Ultra filtration |
| UHT | Ultra high temperature |
| UCF-PC | Ultracentrifugation pellet |
| UV | Ultraviolet |
| UNICEF | United Nations Children's Fund |
| USFDA | United States Food and drug administration |
| Val | Valine |
| Vit A | Vitamin A |
| VAD | Vitamin A deficiency |
| VAD | Vitamin A deficiency |
| v/v/v | Volume by volume by volume |
| $H_2O$ | Water |
| w/v | Weight by volume |
| w/v | Weight by volume |
| w/w | Weight by weight |
| WPC | Whey protein concentrate |
| WHO | World Health Organization |
| α-CN | α-casein |
| α-la | α-lactalbumin |
| $\alpha_{S1}$-CN | $\alpha_{S1}$-casein |
| $\alpha_{S2}$-CN | $\alpha_{S2}$-casein |
| α-TA | α-tocopherol acetate |
| β-CN | β-casein |
| β-lg | β-lactoglobulin |
| κ-CN | κ-casein |

## Abstract

Micronutrient malnutrition is a serious threat to the health and productivity of billions of people worldwide. Among micronutrients, vitamin A deficiency (VAD) is the second largest cause of global blindness next to cataracts. Food fortification is considered as a suitable strategy to combat micronutrient deficiency. Incorporation of fat soluble vitamins to low fat foods is still a challenging task as these are highly sensitive to oxidation, hence require stabilization. The stability and solubility of fat soluble vitamins can be improved by different methods i.e. encapsulation, use of carrier vehicles and emulsification. Complexation of vitamin with different milk protein may help to address the lower solubility and stability of these vitamins. Different milk protein-Vitamin A (Vit A) complexes were prepared using native (unmodified) and modified (succniylated) milk proteins. Level of succinic anhydride was optimized on the basis of maximum degree of succinylation for the preparation of succinylated sodium caseinate (SNaCas) and succinylated milk protein concentrate (SMPC). Native milk protein-Vit A complexes were prepared using sodium caseinate (NaCas), whey protein concentrate (WPC) and milk protein concentrate (MPC). Modified milk protein-Vit A complexes were prepared using SNaCas and SMPC. Reassembled milk protein-Vit A complexes were prepared using NaCas and SNaCas. Native and modified milk protein-Vit A complexes were prepared by stirring (ST) and sonication. Analytical conditions were optimized for estimation of total and unbound vitamin A in milk protein-Vit A complexes. Milk protein-Vit A complexes i.e. NaCas-VA ST, SNaCas-VA ST, RNaCas-VA and RSNaCas-VA were selected for further studies on the basis of vitamin A binding ability of milk proteins and solubility of milk protein-Vit A complexes. Physicochemical characteristics such as particle size, zeta potential, turbidity analysis and tryptophan intensities confirmed structural modification of both native and modified proteins upon complex formation with vitamin. Complexes showed higher vitamin A stability when packed in aluminium laminate pouches as compared to microcentrifuge tubes (virgin polypropylene). Free vitamin A (oily form) showed lowest vitamin A stability followed by NaCas-VA ST, SNaCas-VA ST, RNaCas-VA and RSNaCas-VA at different storage temperatures i.e. -20, 4 and 37°C. Stability of milk protein-Vit A complexes were ascertained at three different pH (3.0, 5.0 and 7.0). *In-vitro* bioavailability of vitamin A was higher for milk protein-Vit A complexes as compared to free vitamin A (oily form). Fortification of milk with free vitamin A (oily form) and milk protein-Vit A complexes did not have significant influence on sensory and physicochemical properties of fortified milk. Vitamin A stability was lowest in sterilized milk followed by boiled and pasteurized milk. The stability of vitamin A was lowest for free vitamin A (oily form) fortified milk followed by NaCas-VA ST, RNaCas-VA, RSNaCas-VA fortified milk and control (unfortified) milk. Pasteurized milk fortified with vitamin A showed higher stability in transparent glass bottles as compared to LDPE pouches during storage at 4-7°C for 7 days, however, higher stability of vitamin A was observed in LDPE pouches as compared to transparent glass bottles during exposure to different light intensities i.e. 1485, 2970 and 4455 lux. Milk protein-Vit A complex fortified milk showed higher *in-vitro* bioavailability of vitamin A as compared to free vitamin A (oily form) fortified milk.

सारांश

सूक्ष्मपोषक कुपोषण दुनिया भर के अरबों लोगों के स्वास्थ्य और उत्पादकता के लिए एक गंभीर खतरा है। विटामिन ए की कमी मोतियाबिंद के बाद वैश्विक दृष्टिहीनता का दूसरा सबसे बड़ा कारण है। खाद्य सम्पूरण सूक्ष्म पोषक तत्व की कमी से निपटने के लिए एक उपयुक्त रणनीति के रूप में माना जाता है| वसा में घुलनशील विटामिन का कम वसा वाले खाद्य पदार्थों में समावेश अभी भी एक चुनौतीपूर्ण कार्य है, क्योंकि ये ऑक्सीकरण के लिए अत्यधिक संवेदनशील हैं इसलिए इन्हें स्थिरीकरण की आवश्यकता है| वसा में घुलनशील विटामिन की स्थिरता और विलेयता में विभिन्न विधियों जैसे एनकैप्सूलेशन, वाहक वाहनों का उपयोग और एमुल्सिफिकेशन द्वारा सुधार किया जा सकता हैं| विटामिन के विभिन्न दूध प्रोटीन के साथ कॉम्प्लेक्स इन विटामिनों के विलेयता और स्थिरता का पता करने के लिए मदद कर सकते हैं| मूल (अनमोडीफायड) और मोडीफायड (सक्सीनाइलटेड) दूध प्रोटीन का उपयोग कर के विभिन्न दूध प्रोटीन-विटामिन ए कॉम्प्लेक्स तैयार किये गए| सक्सीनाइलटेड सोडियम केजीनेट और सक्सीनाइलटेड सोडियम कंसन्ट्रेट की तैयारी के लिए सक्सीनिक एनहाइड्राइड का स्तर सक्सीनाइलेशन की अधिकतम डिग्री के आधार पर अनुकूलित किया गया था। सोडियम केजीनेट, व्हेय प्रोटीन कंसन्ट्रेट, मिल्क प्रोटीन कंसन्ट्रेट की मदद से मूल मिल्क प्रोटीन- विटामिन ए कॉम्प्लेक्स तैयार किये गए| सक्सीनाइलटेड सोडियम केजीनेट और सक्सीनाइलटेड सोडियम कंसन्ट्रेट का उपयोग करके संशोधित मिल्क प्रोटीन- विटामिन ए कॉम्प्लेक्स तैयार किये गए| रीअसेम्बल्ड मिल्क प्रोटीन- विटामिन ए कॉम्प्लेक्स सोडियम केजीनेट और सक्सीनाइलटेड सोडियम केजीनेट का उपयोग करके बनाया गया| मूल और संशोधित मिल्क प्रोटीन- विटामिन ए कॉम्प्लेक्स स्टीररिंग और सोनिकेशन करके बनाये गए| मिल्क प्रोटीन- विटामिन ए कॉम्प्लेक्स में कुल और अनबाउंड विटामिन ए के आकलन के लिए कंडीशन्स ऑप्टिमाइज़ की गयी| दूध प्रोटीन की विटामिन ए बाइंडिंग क्षमता और मिल्क प्रोटीन- विटामिन ए कॉम्प्लेक्स की विलेयता के आधार पर मिल्क प्रोटीन- विटामिन ए कॉम्प्लेक्स जैसे NaCas-VA ST, SNaCas-VA ST, RNaCas-VA and RSNaCas-VA को आगे के अध्ययन के लिए चुना गया| भौतिक विशेषताओं जैसे कण आकार, जीटा पोटेंशल मलिनता विश्लेषण और ट्रीप्टोफैन तीव्रता ने दोनों मूल और संशोधित प्रोटीन विटामिन के साथ जटिल गठन पर संरचनात्मक संशोधन की पुष्टि की | माइक्रोसेंट्रीफ्यूज ट्यूब की तुलना में एल्यूमीनियम के पाउच में पैक कॉम्प्लेक्स उच्च विटामिन ए स्थिरता पायी गयी| विभिन्न भंडारण तापमान यानी -20, 4 और 37 °c पर फ्री विटामिन ए (तेल प्रपत्र) के बाद सबसे कम विटामिन ए स्थिरता NaCas-VA ST, SNaCas-VA ST, RNaCas-VA and RSNaCas-VA में पायी गयी| मिल्क प्रोटीन- विटामिन ए कॉम्प्लेक्स की स्थिरता तीन विभिन्न पीएच पर (3.0, 5.0 और 7.0) पर देखी गयी और उच्चतम स्थिरता 7.0 पीएच पर पायी गयी| विटामिन ए की इन-विट्रो पशुमूल फ्री विटामिन ए (तेल प्रपत्र) की तुलना में मिल्क प्रोटीन- विटामिन ए कॉम्प्लेक्स में ज्यादा पायी गयी| फ्री विटामिन ए (तेल प्रपत्र) और मिल्क प्रोटीन- विटामिन ए कॉम्प्लेक्स की दूध सम्पूरण करने पर संवेदी और भौतिक गुणों पर महत्वपूर्ण प्रभाव नहीं है। विटामिन ए की स्थिरता स्टेरलाइज़्ड दूध के बाद उबले और पाश्चरइज़्ड दूध में सबसे कम थी| विटामिन ए की स्थिरता फ्री विटामिन ए सम्पूरित दूध के बाद NaCas-VA ST, RNaCas-VA, RSNaCas-VA सम्पूरित दूध में सबसे कम पायी गयी| विटामिन ए सम्पूरित पाश्चरइज़्ड दूध की स्थिरता 4-7 डिग्री सेल्सियस पर भंडारण के दौरान एलडीपीई पाउच की तुलना में कांच की बोतलों में ज्यादा पायी गयी, तथापि, विटामिन ए की स्थिरता कांच की बोतलों की तुलना में एलडीपीई पाउच में विभिन्न प्रकाश तीव्रता यानी 1485, 2970 और 4455 लक्स को प्रदर्शन के दौरान ज्यादा पायी गयी। फ्री विटामिन ए सम्पूरित दूध की तुलना में मिल्क प्रोटीन- विटामिन ए कॉम्प्लेक्स की उच्च इन-विट्रो पशुमूल पायी गयी|

# CHAPTER – 1

## Introduction

# INTRODUCTION

Nutritional status of a population is one of the most important factors in determining the quality and productivity, which in turn will affect national productivity. 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. These deficiencies may cause impaired physical growth and cognitive development, 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).

Vitamins are essential for normal health and development. 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 VAD (McGuire and Galloway 1994).

Pharmaceutical supplementation, dietary diversification and food fortification are some useful strategies for controlling micronutrient malnutrition. Among these, fortification of food with micronutrients can be an effective strategy to combat micronutrient deficiencies in developing countries (Allen *et al*. 2006). Government of India is committed to tackle VAD through milk fortification. FSSAI approved the fortification of toned/double toned/skimmed milk with vitamin A and vitamin D. Fortified milk should contain minimum 770 IU of vitamin A and 550 IU of vitamin D per liter of milk (FSSR 2016). Incorporation of fat soluble substances in functional foods is a difficult challenge, especially in fat free and low fat foods, which are

growing in demand. Most of these hydrophobic compounds e.g. fat soluble vitamins are highly sensitive towards oxidation by light and heat, and thus require stabilization in an aqueous medium and protection against deteriorating factors (Zimet *et al.* 2011). Nano-vehicles are formed to facilitate the dissolution and protection of hydrophobic neutraceuticals (Uversky 2002). To this end, significant advances have been made, in respect to the formation and rational design of particulate based delivery system. Commercially available forms of vitamin A are both oil soluble and water soluble/miscible. Oily forms need homogenization for incorporation in the product and water soluble forms were less stable due to dissolution of protective coating, leaving the vitamin susceptible to degradation. Moreover, vitamins microencapsulated with polysaccharides exhibit reduced release of vitamins under simulated intestinal conditions as compared to protein-vitamin complexes (Teng *et al.* 2013). Therefore, milk proteins may be an effective vehicle for complex formation with vitamins and controlled release of vitamins under simulated intestinal conditions.

Milk proteins are widely accepted as alimentary elements appropriate for delivery of bioactive compounds, since milk is remarkable component of human diet (Livney 2010). Thus, various milk proteins (α-casein, ß-casein, caseinate, reassembled caseinate, ß-lactoglobulin, bovine serum albumin, lactoferrin etc.) have received considerable attention as potential delivery vehicles for various bioactive compounds and micronutrients (Tavares *et al.* 2014). Milk proteins have important functional properties such as the ability to bind hydrophobic molecules, interact with other biopolymers, stabilize emulsions, form gels and to some extent, retard oxidation. Due to these properties, milk proteins can be an ideal material for the entrapment and delivery of micronutrients and bioactive compounds (Livney 2010). Different milk proteins including whey protein and casein fractions have been evaluated for binding with different vitamins i.e. tocopherol, vitamin A, vitamin D, folic acid etc. (Bourassa *et al.* 2013; Esmaili *et al.* 2011; Forrest *et al.* 2005; Haratifar and Corredig 2014; Hasni *et al.* 2011; Pérez *et al.* 2014a; Perez *et al.* 2014b; Sáiz-Abajo *et al.* 2013; Semo *et al.* 2007; Zhang *et al.* 2014; Zimet *et al.* 2011). Milk protein comprise of 80% casein and 20% whey protein (Fox and McSweeny 2003). Casein

has very strong tendency to associate, which is convenient for nanoencapsulation. Major problem associated with the use of milk proteins for delivery of vitamins is their solubility; however, this can be improved by succinylation of milk proteins. Succinylation results in incorporation of additional carboxylic group to free amino and hydroxyl groups of amino acid (especially Lys) which further modifies some physico-chemical and biochemical properties of proteins (Schwenke *et al.* 1981). These modified proteins can also be used as a carrier for different bioactive compounds and micronutrients. Succinylation also result in unaltered amino acid composition with only slight decrease in lysine content of protein. Major amino acid involved in the interaction of protein with hydrophobic compounds are Tyr, Phe, Trp, Leu and Val. Succinylation affect mainly Lys, other amino acid involved in hydrophobic interaction may not be affected. Therefore, modified protein might be able to bind hydrophobic compounds to the same extent as that of unmodified protein (Thompson and Reyes 1980). Succinylation also increased the hydrophobicity of milk protein due to conformational changes which resulted in greater exposure of hydrophobic core (Lakkis and Villota, 1992). USFDA (United States Food and drug administration) gave generally recognized as safe (GRAS) status to succinic anhydride for miscellaneous and general purpose usage with no limitation other than good manufacturing practices (Smith and Hong-Shum 2011). Succinic anhydride can be used in the production of starch sodium succinate at the level not to exceed 4% as per Food Chemicals Codex (1981). Reports are available in literature regarding the binding of different milk proteins (phosphocasein micelle, reassembled casein micelles, ß-lactoglobulin, whey protein, α-casein, ß-casein etc.) to hydrophobic compounds (curcumin, vitamin $D_2$, vitamin $D_3$, ß-carotene, ω-3 polyunsaturated fatty acid, retinyl acetate, α-tocopherol, green tea flavanoids, folic acid etc.) but to our knowledge no attempt have been made for the preparation, characterization, stability and application of milk protein (native/succinylated)-vitamin A complexes and *in-vitro* bioavailability of vitamin A from these complexes. Therefore, the present study was envisaged with the following objectives:

**Objectives:**

1. Preparation of milk protein- vitamin A (Vit A) complexes.
2. Physicochemical characterization, stability and *in-vitro* bioavailability of milk protein-Vit A complexes
3. Evaluation of sensory and physico-chemical attributes of milk protein-Vit A complex fortified milk and *in-vitro* bioavailability of added vitamin A

# CHAPTER –2

# Review of Literature

## 2.1 Micronutrient malnutrition:

Micronutrients are nutrients required by humans and other living creatures in small quantities to orchestra a whole range of physiological functions, which the organisms itself cannot produce (Lieberman and Bruning 1990). Vitamin and mineral deficiencies affect more than 2 billion people worldwide and are especially prevalent in developing countries including India (Ezzati *et al.* 2004). Conditions arising due to vitamin and mineral deficiencies account for 11 percent of the global burden of the disease and VAD ranks among the 15 leading causes of global burden (Black *et al.* 2008). Micronutrient malnutrition is a serious problem among children in developing countries, caused by lack of food and improper diet. Deficiency of micronutrients such as iron, iodine and vitamins leads to malnutrition (Muller and Krawinkel 2005). Malnutrition is an underlying cause of death for 2.6 million children each year (Black *et al.* 2008) and one third of under-five deaths (UNICEF 2011). One in four of the world's children are stunted (Thamilini 2015) and in developing countries this is as high as one in three (Black *et al.* 2008).

Out of the world population of seven billion, two billion suffer from micronutrient deficiencies (Global nutrition report 2016). Globally, micronutrient deficiencies of greatest public health significance are those of iron, vitamin A, iodine and vitamin D. Insufficient dietary intake of these essential nutrients result in long lasting effects on physical growth, cognitive development, immune response and productivity. In our country more than half the population across any age group consume less than 50% of their daily needs of iron, zinc, vitamin A, folate and other B-vitamins (NNMB 2003).

About one third of the world's population was reported to be deficient in one or more micronutrients. Consumption of fruits, vegetables, meat and eggs which are considered as richest sources of vitamin and minerals is very low among the Indian population. Vitamins and minerals deficiencies often go unnoticed, hence called "hidden hunger" (NSSO 2011).

### 2.1.1 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).

VAD affects an estimated 190 million pre-school aged children and 19 million pregnant and breastfeeding women globally (WHO 2009). The National Nutrition Monitoring Bureau (NNMB) survey and a report of the expert group of Indian Council of Medical Research (ICMR) had stated that India has a very high burden of vitamin A and D deficiencies, amongst both young children and adults particularly those in urban areas who are physically less active and have a very limited exposure to sunlight (http://foodfortificationrajasthan.in/Milk_FF.aspx, 2016). In India, more than a quarter of the world's vitamin A deficient preschool children reside who suffer from subclinical VAD and one-third of the preschool children show clinical signs and symptoms of VAD (WHO 2009).

### 2.1.2 Vitamin A and its deficiency

Among micronutrients, VAD is most lethal. 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 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 VAD. 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. 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).The risks become less in older children, but VAD reduces overall immunity and makes children susceptible to diseases like measles and diarrhea. United Nations Children's Fund (UNICEF) estimated that VAD is a public health concern in 75 countries of Asia and Africa. Horton *et al.* (2008) reported that VAD is a public health concern if the mortality rate of children below five years is greater than 70/1,000. This means that the death of more than 70 children among 1,000 indicates VAD. In 1970, the mortality rate of children under five years in India was 130/1,000, thereby making VAD a public health concern was important (Hindu Health, December 8, 2001). In pregnant women, VAD causes night blindness and may increase the risk of maternal mortality (Allen *et al.* 2006). 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 VAD 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.

### 2.1.3 Strategies for the control of micronutrient malnutrition

The control of micronutrient 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 (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, 2016) and the draft of National Health Research Policy (NHRP 2010) identified micronutrient malnutrition as a major concern. Countries may lose two to three percent of their Gross Domestic Product (GDP) as a result of iron, iodine, and zinc deficiencies (http://www.gainhealth.org/knowledge-centre/fast-facts-malnutrition/, 2016). In India, loss due to micronutrient deficiencies costs 1% of its GDP which is ultimately a loss of Rs. 27,720 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 (Hoddinott *et al.* 2012). 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).

Policy and programme responses include food-based strategies that can be used to improve micronutrient malnutrition management:

a) Supplementation with pharmaceuticals dispensed in adequate forms

b) Fortification of food or water with nutrients

c) Consumer education and dietary change through the expansion of the demand for, and supply of, nutrient rich food.

(Salgueiro *et al.* 2007)

## 2.2 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. The Codex *General Principles for the Addition of Essential Nutrients to Foods* (Codex 1987) defines "fortification" 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". Fortification refers to addition of vitamins and minerals to commonly consumed foods in order to prevent

nutritional deficiencies. Minute quantities of missing nutrients in the daily diet such as vitamin A, iron and iodine are added to commonly consumed foods e.g. milk, wheat flour, rice and oil to enhance the nutrient quantity of food (FSSAI 2016). Food fortification differs from other programmes as it involves the addition of nutrients to foods in respect to its nutritional intervention with a specifically defined target and fortified food products are expected to become a main source of the specifically 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. This may 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).

(Allen *et al.* 2006)

- Fortification technology is simple to use and easy to implement
- Cost of fortification is minimal
- Nutrients regularly used in staple food fortification prevent diseases, strengthen the immune system and improve productivity and cognitive development.

(FSSAI 2016)

### 2.2.1 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); allow food manufacturers to voluntarily fortify foods available in the market place (market-driven fortification); fortify foods at household level (household fortification) and/or fortification of the staple foods by breeding and genetic modification of the plants (bio 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). Household and bio fortification are also gaining importance (Nestel 2003).

#### 2.2.1.1 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 (Allen *et al.* 2006). Although this statement may be true in many cases, it depends on the existence of several pre conditions. 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
- Nutrition and health claims that promote healthy practices.

(www.a2zproject.org/pdf/Food-Fortification-Formulator.pdf, 2016)

#### 2.2.1.2 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).

#### 2.2.1.3 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).

#### 2.2.1.4 Household fortification

Efforts are underway in a number of countries to develop and test practical ways of adding micronutrients to foods at the household level, in particular, to complementary foods for young children. In effect, this approach is a combination of supplementation and fortification, and has also been referred to as “complementary food supplementation” (Nestel 2003).

#### 2.2.1.5 Bio fortification of staple foods

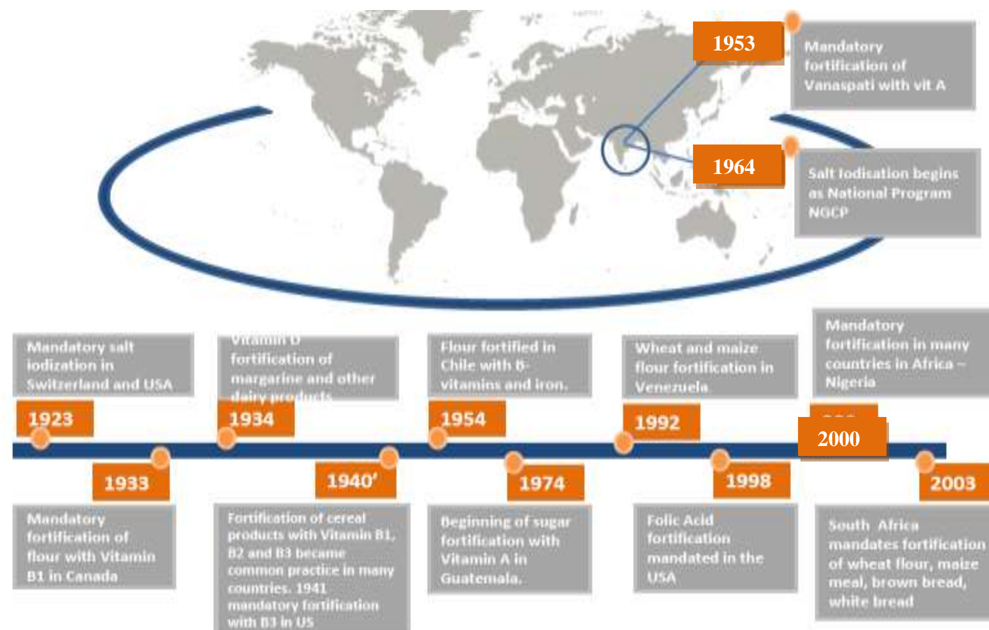
It involves the breeding and genetic modification of plants so as to improve their nutrient content and absorption. Certain cereals (maize, millet and sorghum) and legumes (lentils, chickpeas, peas and soybeans) are known for their high iron content, various varieties of carrot and sweet potatoes for their favourable β-carotene levels, and maize for their low phytate content, these can be used as vehicles for fortification (Beyer 2002; Lucca *et al.* 2002).

### 2.2.2 History of food fortification

Fortification is a century old technology to address vitamin and mineral deficiencies. It has a long history of use in industrialized countries for the successful control of deficiencies of vitamins A, D, several B vitamins (thiamine, riboflavin and niacin), iodine and iron. Salt iodization was introduced in the early 1920s in both Switzerland (Burgi 1990) and the USA (Marine and Kimball 1920) and has since expanded progressively all over the world to the extent that iodized salt is now used in most countries. In India, salt fortification with iodine was initiated in 1964 (FSSAI 2016). Darnton-Hill and Nalubola (2002) have identified at least 27 developing countries that could benefit from programmes to fortify one or more foods. Fortification is often more cost-effective than other strategies, especially if the technology already exists and if an appropriate food distribution system is in place (Horton 1999).

India, Mexico and Chile initiated fortification of margarine and vanaspati with vitamin A in 1953, 1974 and 1997, respectively. Central America and Philippines initiated fortification of sugar with vitamin A in 1974 and 2000, respectively. Denmark and India initiated fortification of margarine with vitamin D in 1918 and 1953, respectively. Indonesia and New Zealand initiated fortification of vanaspati with vitamin D in 1996 and 2007, respectively. In 2002, Mexico initiated fortification of milk with vitamin D. Canada, USA, Chile and Australia initiated the fortification of wheat flour with iron, vitamin $B_1$, $B_2$, niacin and folic acid in 1933, 1941, 1954 and 2009, respectively. Costa Rica and Philippines initiated the fortification of rice with iron, vitamin $B_1$, $B_2$, niacin and folic acid in 1991 and 2001, respectively. In 1998,

Indonesia initiated the fortification of wheat flour with zinc. In 1991, Costa Rica initiated the fortification of rice with zinc.




(http://www.nams-india.in/downloads/cmeevents/nfi03.pdf, 2016)

**Fig 2.1: Global timeline for food fortification**

In 1938, USA introduced mandatory fortification of corn, wheat and rice products with iron and B complex vitamins. In 1949, Canada introduced mandatory iodization of salt. In Australia, it is mandatory to fortify edible oil, spreads and margarine with vitamin D and wheat flour used for bread with thiamine and folic acid. It is also mandatory to use iodized salt for bread making. In 2016, wheat, maize or rice flour fortification with at least iron or folic acid was mandated in 86 countries (Food Fortification Initiative 2016).

Population of USA and Canada gets quarter of its daily iron intake from fortified flour. Double fortification of milk and margarine with vitamin A and D helped in the elimination of rickets from many European countries and North America. In USA, 99% of milk supply is fortified with 10 µg of vitamin D per litre (Food Nutrition Initiative 2016). In 2015, 13.2% of neural tube defects were prevented in 58 countries by practicing mandatory fortification of flour with folic acid (Arth *et al.* 2016). In 1970s, sugar was fortified with vitamin A and it was effective in reducing VAD in the population of Central America (Dary and Mora 2002). Due to iron

fortification, anemia was reduced from 19% to 4% in children, and from 18% to 10% in women of Costa Rica (Martorell *et al.* 2015).

The National Prophylaxis Programme against Nutritional blindness (NPPNB) due to VAD was initiated in 1970 with the specific aim of preventing nutritional blindness due to keratomalacia. The programme was launched as an urgent remedial measure to combat the unacceptably high magnitude of xerophthalmic blindness in the country seen in the 1950s and 1960s. Clinical VAD has declined drastically during the last 40 years. Also, indicators of child health have shown substantial gains in different states of the country (Kapil and Sachdev 2013). The NPPNB due to VAD, of the Government of India, has a provision for administering mega doses of vitamin A. It recommends for at least nine doses of vitamin A to be given to all children aged 9 to 59 months. The first dose of 100,000 International Unit (IU) is administered with measles vaccination at 9 months and subsequent doses of 200,000 IU each, every six months (MOHFW 1991; MOHFW 2006). Vitamin A supplementation programme for children under five has been running for several decades. As per the National Family Health Survey (NFHS) III (2005-06), only one in four children aged 12–35 months received the six monthly vitamin A supplement in the six months. This figure drops further, to only 18%, among children aged 6–59 months (NFHS 2007). State wise report of NFHS IV (2015-16) showed that 65-80% children aged 9–59 months received the six monthly vitamin A supplement in the six months in different states of the country (NFHS 2015). Annual Health Survey 2010–11 showed that at least every second child aged 6–35 months has received a vitamin A supplement in AHS States in the last six months— except in Uttar Pradesh where it is every third child. AHS 2010–11 covered 9 states—UP, Rajasthan, Odisha, Bihar, MP, Uttarakhand, Assam, Jharkhand and Chhattisgarh). These surveys suggest that the challenge of achieving high coverage with completion of all six monthly vitamin A doses (9 months to 5 years), remains an unfinished agenda, especially in states with low coverage rates (Planning Commission 2013). In 2011, WHO reiterated their earlier recommendations supporting the use of high dose vitamin A supplements to reduce child morbidity and mortality in populations where clinical and subclinical VAD is a public health problem (WHO 2011). Twelfth five

year plan of the Government of India focused on the approaches to improve dietary intake of vitamin A that could help in improving the vitamin A status in the long run (Planning Commission 2013).

### 2.2.3 Selection of food vehicle

Selection of a suitable food vehicle and compatible fortificants 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 (Allen *et al.* 2006).

Fortification of food with vitamin A is an approach that nutritionists suggest to overcome this deficiency. Foods which have been successfully fortified with vitamin A include margarine, fats and oils, milk, sugar, cereals and instant noodles with spice mix. In developed countries, vitamin A fortification is limited to milk and milk products, margarine, fat spreads and breakfast cereals. Current levels of fortification of vitamin A are generally considered as safe. Carotenoids can be used as a source of vitamin A. However, the conversion and bioavailability of the carotenoids is known to be affected by the vitamin A status of the individual and by dietary composition (Vir 2011).

Vitamin A and carotene in milk exist in the fat portion; much of the vitamin A activity is removed with the milk fat during the manufacture of reduced fat and nonfat milks. Consequently, reduced-fat and nonfat milks are required to be fortified with chemically derived vitamin A (e.g. retinyl palmitate) to a level found in whole milk or 300 IU (6% Daily Value) per 8-fluid-ounce serving. However, dairy processors are encouraged to continue to fortify low fat milks to the current level of 500 IU of vitamin A per cup (10% Daily Value) or 2,000 IU per quart (Hicks *et al.* 1996). Good manufacturing practices require vitamin A to be suspended in reduced fat and nonfat milks.

### 2.3 Milk as a vehicle for vitamin A 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.

#### 2.3.1 History and successful interventions

Fortification of milk with vitamins commenced in early nineties. USA, UK, Malaysia, Thailand and Mexico initiated fortification of milk with vitamin A in 1923, 1923, 1985, 1993 and 2002, respectively (FSSAI 2016). Vitamin D fortification of milk in the UK is reported to begun in 1923. Several countries have established mandatory fortification of milk, since early in this century (Argentina, Brazil, Malaysia, Mexico, USA etc.). 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 vitamin A, vitamin 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. In 2005, US regulation (Pasteurised Milk Ordinance) stipulated vitamin A fortification in all reduced fat milk products. During 1980s, the Department of Food, Government of India introduced a scheme of fortifying milk with vitamin A at 2000IU/L for toned/double toned milk to prevent

nutrition blindness. The government reimbursed the cost of vitamin A premix to the dairies for fortifying milk for three years, after which dairies were asked to reabsorb the cost. In 1988-89, total quantity of milk fortified with vitamin A was 3.2 million litres per day (NNP 1993). Today, Mother Dairy continues to fortify vitamin A and D in its 9 lakh litres of bulk vended milk per day. Rajasthan cooperative Dairy Federation started fortifying milk in the year 2013 with vitamin A and D and has demonstrated a successful model of providing these essential vitamins to millions of people in Rajasthan.

Recently, Food safety and standards Authority of India (FSSAI) has formulated a comprehensive regulation on fortification of foods namely “Food Safety and Standards (Fortification of Foods) Regulations (FSSR), 2016”. These regulations have set the standards for food fortification and encourage the production, manufacture, distribution, sale and consumption of fortified foods. The regulations also provide for specific role of FSSAI in promotion of food fortification and to make fortification mandatory. Five categories of food are covered under this regulation i.e. wheat flour, rice, milk, oil and salt. FSSAI approved the fortification of wheat flour and rice with iron, folic acid, vitamin $B_{12}$, zinc, vitamin A, thiamin, riboflavin, niacin and pyridoxine, fortification of milk and oil with vitamin A and D and fortification of salt with iodine or double fortified with iron and iodine. Vitamin A fortified in milk, vegetable oil, *atta* and *maida* should be in the form of retinyl palmitate, retinyl acetate and retinyl propionate. In rice, vitamin A has to be fortified as retinyl palmitate (FSSR 2016).

### 2.3.2 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. Vitamins 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.

### 2.3.3 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

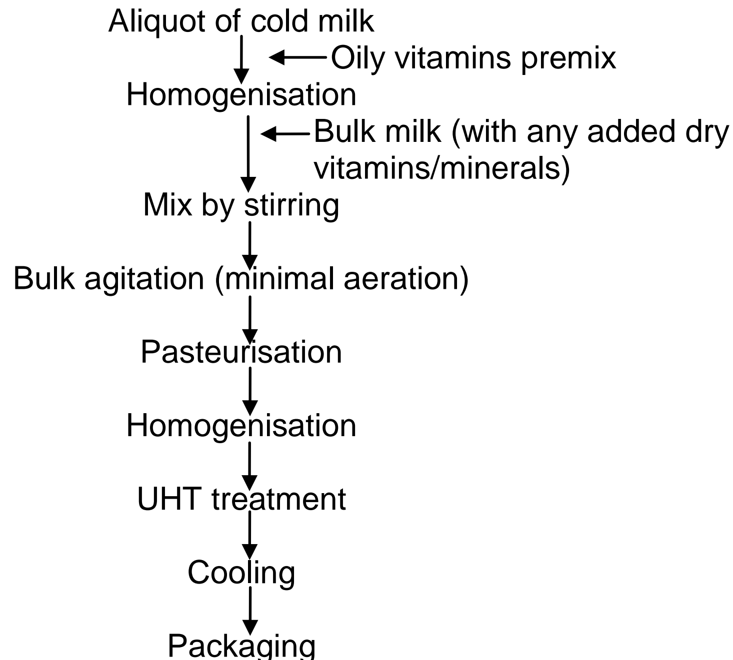



**Figure 2.1: Process flow chart of liquid milk fortification**

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.

### 2.4 Vitamin A fortification

NNMB 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 (NNMB 2003). 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 2.1 illustrates the current status of milk fortification with

vitamins A in different countries. Recently, FSSAI approved the fortification of vitamin A in milk, vegetable oil, wheat flour and rice (FSSR 2016).

**Table 2.1: 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.

### 2.4.1 Physiological significance

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 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).

### 2.4.2 Structure of retinol and its esters

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

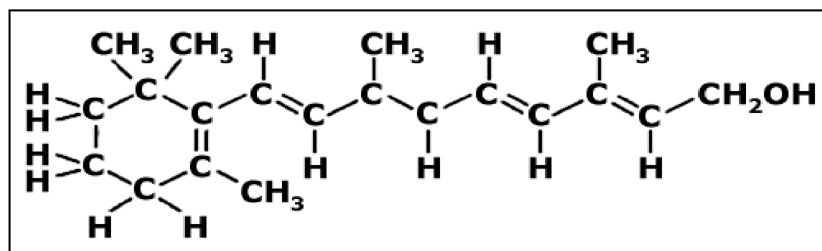



**Figure 2.2: Structure of vitamin A (Retinol)**

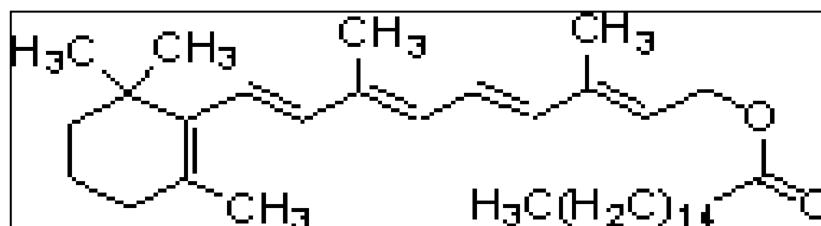



**Figure 2.3: Structure of vitamin A palmitate (Retinyl palmitate)**

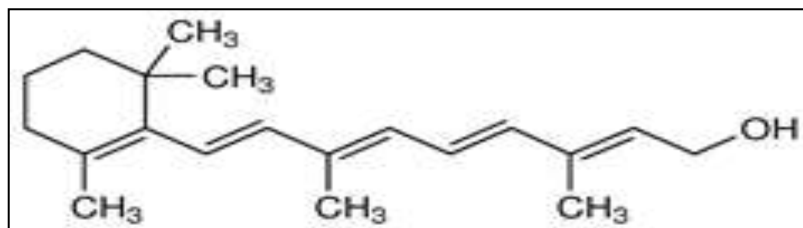



**Figure 2.4: Structure of vitamin A acetate (Retinyl acetate)**

### 2.4.3 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 VAD. Vitamin A is required for healthy vision and other

**Table 2.2: Recommended dietary allowance of vitamin A for Indians**
(Retinol equivalents, RE, μg/d)

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

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 2.2).

### 2.4.4 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 diarrhea diseases. Subclinical VAD is also associated with an increased risk of child mortality, especially from diarrhea and measles (Allen *et al.* 2006).

In general, three 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 in 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 in the developing nations (Favaro *et al*. 1991; Lee *et al*. 2000).

### 2.4.5 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).

### 2.4.6 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, retinyl palmitate, along with

provitamin A (β-carotene), are the main commercial forms of vitamin A that are available for use as food fortificants.

**Table 2.3: 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)

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 2.3 shows the commercially available forms of vitamin A. 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.

### 2.4.7 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

**Table 2.4: 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) |

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 2.4 illustrates different HPLC methods used for vitamin A analysis.

### 2.4.8 Stability

Vitamins are sensitive to heat, light and humidity, as well as oxidising and reducing agents to different degrees. Moisture contents in excess of about 7-8% in a food are known to adversely affect the stability of vitamin A. Beyond the critical moisture content there is a rapid increase in water activity which permits various deteriorative reactions to occur. Repeated heating, as may be experienced with food processing, is known to significantly degrade vitamin A (Labuza *et al.* 1970).

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). 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.

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. However, in low fat milk, microwave heating for two 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. No substantial loss was observed in vitamin A content of fortified toned

milk at refrigerated temperature (5-7ºC) during storage of 48 h. 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 h which increased to 10.2% at the end of 96 h. 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 (3% fat) than in skim milk. Increasing light intensity increased the loss of vitamin A. Increased fat percent had a protective effect on vitamin A. Higher amount of retinyl palmitate was retained in 2% and whole milk (3% fat) 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 nutrients, taste and flavor of milk better than plastic containers 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 high fat 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 sensitive to light and degraded more rapidly than indigenous vitamin A when concentrated oil solutions of vitamin A and emulsifier were used for fortification. Storage can affect the concentration of vitamins added to milk. In nonfat and low fat milks, added vitamin A gradually decreases during normal storage of milk at 40°F in the dark. However, this vitamin is rapidly destroyed when milk is exposed to sunlight in transparent glass bottles or translucent plastic containers. Thompson and Erdody (1974) reported that vitamin A added to milk is more sensitive to light than native vitamin A; losses of 50% of the added vitamin A occurred during 3 h of exposure to fluorescent light at ambient temperature compared with no loss of native vitamin A even with 5 h of exposure. Researchers have investigated several factors that influence the stability of vitamin A in milk. This light-induced destruction of vitamin A depends on the intensity and wave length of the light and the milk source. Vitamin A destruction can be retarded by the use of amber or brown glass bottles, pigmented plastic containers formulated with specific light barriers and colored paper cartons. Vitamin A losses of 8% to 31% were found in 2% low fat milk exposed to 200 foot candles of fluorescent light for 24 hours in opaque plastic containers. These losses were virtually eliminated by use of pigmented containers or gold shields over fluorescent tubes (Bector and Rani 1998).

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.

### 2.4.9 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 (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 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, 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 (Granado *et al.* 2006).

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. Garrett *et al.* (2000) estimated the bioavailability of carotenoids from fresh stir-fried vegetables using an *in-vitro* digestion model. Digesta was prepared by treating the sample with gastric, duodenal and bile solutions. Micellar carotenoids were absorbed through the intestinal cells. Micellar fraction was separated from the digesta by centrifugation at 167000 g for 95 min at 4°C and further filtration through 0.22 µm syringe filters. Hornero-Mendez and Mínguez-Mosquera (2007) used 47000 rpm for 50 min at 4°C for separation of micellar fraction from digesta. Ryan *et al.* (2008) used 53000 rpm for 95 min for separation of micellar fraction from digesta. Veda *et al.* (2006) evaluated the suitability of membrane filtration method for separation of micellar fraction as compared to ultracentrifugation method and reported that bioaccessibility of ß-carotene estimated by membrane filtration method differed non significantly from ultracentrifugation method.

**2.5 Milk proteins**

Milk contains approximately 3.5% protein, which falls in to two principle categories. The first ones are caseins, which constitute about 80% of the total milk

protein and the remaining 20% are whey proteins, which remain in solution at pH 4.6 (Walstra and Jenness 1984). Table 2.5 shows some of the structural and chemical characteristics of the major milk proteins.

**Table 2.5: Some structural and chemical characteristics of milk proteins**

| Properties | Whey protein | | | Casein | | | |
|---|---|---|---|---|---|---|---|
| | **β-lg** | **α-la** | **BSA** | **$\alpha_{S1}$-** | **$\alpha_{S2}$-** | **β-** | **κ-** |
| Molecular weight | 18362 | 14194 | 65000 | 23612 | 25228 | 23980 | 19005 |
| Total residues | 162 | 123 | 581 | 199 | 207 | 209 | 169 |
| Apolar residue (%) | 34.6 | 36 | 28 | 36 | 40 | 33 | 33 |
| Isoionic Point | 5.2 | 4.2-4.5 | 5.3 | 4.96 | 5.27 | 5.2 | 5.54 |
| Proline residues | 8 | 2 | 28 | 17 | 10 | 35 | 20 |
| Phosphoserine residue | 0 | 0 | 0 | 8-9 | 10-13 | 5 | 1 |
| Disulphide bonds | 2 | 4 | 17 | 0 | 2 | 0 | 2 |
| Thiol group | 1 | 0 | 1 | 0 | 0 | 0 | 0 |

Abbreviations used: β-lg= β-lactoglobulin; α-la= α-lactalbumin; BSA= bovine serum albumin; $\alpha_{S1}$-CN=$\alpha_{S1}$-casein; $\alpha_{S2}$-CN=$\alpha_{S2}$-casein; β-CN=β-casein; κ-CN=κ-casein

(Kinsella *et al*. 1989)

### 2.5.1 Casein

The caseins are synthesized in the mammary gland. Compositionally, the hallmark of the casein is ester-bound phosphate (Ng-Kwai-Hang 2003a). All caseins contain sites of phosphorylation with a unique sequence –Ser-X-Y-, where X = any amino acid and Y = Glu or Ser-P. All of the phosphorylation sites are located in clusters (Wong *et al*. 1996). This cluster of the phosphoserine residues is particularly apparent and this has a marked influence on the metal binding properties of the caseins. Caseins are classified according to their primary structure: $\alpha_{S1}$-, $\alpha_{S2}$-, β- and κ-CN.

#### 2.5.1.1 $\alpha_{S1}$-CN

$\alpha_{S1}$-CN can be visualized as a loose flexible polypeptide chain. The flexible nature of the polar domain causes the molecular dimensions to be very sensitive to ionic strength and to binding of ions, particularly protons ($H^+$) and $Ca^{2+}$ (Swaisgood 1985). It binds about 8 moles of $Ca^{2+}$ per mole of protein near pH 7.0, probably to ester phosphate groups. It aggregates and precipitates at very low concentration of $Ca^{2+}$ (7mM of $Ca^{2+}$, 28mM of NaCl) (Walstra and Jenness 1984). Intermolecular interactions between hydrophobic domains lead to self association or association with other caseins

(Swaisgood 1985). Self association of $\alpha_{S1}$-CN depends markedly on concentration, pH, ionic strength and kind of ion in the medium but it is relatively independent of temperature (Walstra and Jenness 1984).

#### 2.5.1.2 $\alpha_{S2}$-CN

Among the caseins, $\alpha_{S2}$-CN is the least hydrophobic (Ng-Kwai-Hang 2003a). $\alpha_{S2}$-CN has a remarkable dipolar structure with a concentration of negative charges near the N-terminus and positive charges near C-terminus. It binds $Ca^{2+}$ strongly and more sensitive to precipitation by $Ca^{2+}$ than $\alpha_{S1}$-CN. It self associates at neutral pH in the absence of $Ca^{2+}$ and the association depends markedly on ionic strength ((Walstra and Jenness 1984). Both $\alpha_{S1}$- and $\alpha_{S2}$-CN are precipitated by 6mM $Ca^{2+}$ at all temperatures (Wong *et al.* 1996).

#### 2.5.1.3 β-CN

It has no cysteine residues and high proportion of proline (35 residues), which has a profound effect on its structure (Ng-Kwai-Hang 2003a). β-CN has a strong negatively charged N-terminal region while the rest of the molecule is very hydrophobic and has no net charge (Swaisgood 2003). Consequently, this molecule is very amphiphathic with a polar domain comprising one-tenth of the chains but carrying one-third of the total charge and a hydrophobic domain consisting of the C-terminal three-fourth of the molecule. The outstanding characteristic of the association of β-CN in both the absence and presence of $Ca^{2+}$ is its strong dependence on temperature (Walstra and Jenness 1984; Swaisgood 1985). β-CN tightly binds about 5 $Ca^{2+}$ per mole, consistent with its ester phosphate content (Walstra and Jenness 1984).

#### 2.5.1.4 κ-CN

κ-CN has 2 cysteine residues and serine residues and is the only protein of the casein family that is glycosylated (Creamer 2003). It has an amphipathic character, which encourages it to form micelles in solution. It plays a crucial role in stabilizing the casein micelles in milk and after enzymatic cleavage, destabilizing the colloidal casein system. κ-CN does not contain clusters of phosphoserine residues in its polar domain

as do the calcium-sensitive caseins; hence it does not bind as much $Ca^{2+}$ (1-2 moles $Ca^{2+}$ per mole of protein) and is not sensitive to precipitation by $Ca^{2+}$ (Swaisgood 1985).

**Casein hydrophobicity**

Caseins are generally considered as hydrophobic molecules, however, their amino acid composition indicates that they are not particularly so; in fact, some are more hydrophilic than the whey protein, β-lg. Caseins have high surface hydrophobicity, in contrast to the globular whey proteins, in which the hydrophobic residues are buried as much as possible within the molecule, with most of the hydrophilic residues exposed on the surface, owing to the relative lack of secondary and tertiary structures in the caseins, such an arrangement is not possible, and hence the hydrophobic residues are rather exposed. Thus, the caseins are relatively small, relatively hydrophobic, amphipathic, randomly or flexibly structured molecules, with relatively low levels of secondary and tertiary structures (Fox and McSweeney 1998).

### 2.5.2 Whey Proteins

The term whey proteins refer to milk proteins remaining in the serum (or whey) after precipitation of the caseins from milk at pH 4.6 at 20°C. This fraction accounts for approximately 20% of total protein. Unlike caseins, whey proteins are globular and have more organized secondary and tertiary structure (Ng-Kwai-Hang 2003b). They exist as discrete molecules with varying numbers of disulfide cross links. Compared to caseins, these proteins are more heat sensitive, less sensitive to calcium, can engage in thiol disulfide interchange and form disulfide-linked dimers or polymers, e.g. with κ-CN (Kinsella 1984). There are four major proteins, denoted as β-lactoglobulin (β-lg), α-lactalbumin (α-la), serum albumin and immunoglbulins. These account for >95% of the non-casein proteins. β-lg and α-la are synthesized in the mammary gland whereas serum albumin is transported to the mammary gland via the blood serum (Ng-Kwai-Hang 2003b).

#### 2.5.2.1 β-lg

β-lg accounts for more than 50% of the total whey proteins in bovine milk and about 10% of the total milk proteins in cow's milk (Creamer 2003). It has a monomeric molecular weight of 18300 Da (Kinsella 1984; Walstra and Jenness 1984). The primary

structure of β-lg consists of 162 amino acids. It has 5 cysteine residues capable of forming disulphide bonds between residues 66-160, 119-121 or 106-119 (Ng-Kwai-Hang 2003a). It has a single fee thiol (R-SH) group which is of great importance for changes occurring in milk during heating since it facilitates thiol/disulphide (R-SH/S-S) interchange reactions which allows the formation of new structures or intermolecular disulfide-bonded dimmers and polymers (Kinsella 1984). It is also involved in reactions with other proteins notably κ-CN and α-la upon heating (Walstra and Jenness 1984).

β-lg act as a carrier for retinol (vitamin A). β-lg can bind retinol in a hydrophobic pocket, protect it from oxidation and transport it through the stomach to the small intestine where the retinol is transferred to a retinol-binding protein, which has a similar structure to β-lg. β-lg is capable of binding many hydrophobic molecules and hence its ability to bind retinol may be incidental (Fox and McSweeny 1998).

**2.5.2.2 α-la**

α-la is the second most abundant of the whey proteins accounting for 20% of them (2 to 5% of the skim milk total protein) (Kinsella 1984; Wong *et al.* 1996). It has a sequence of 123 amino acids with 8 cysteine residues, which are connected by 4 disulphide bridges between positions 6-120, 28-111, 61-77 and 73-91. There are four inter-chain disulphide bonds, but no sulphydryl groups are present in α-la, hence it is relatively heat stable (Kinsella 1984). α-la contains tightly bound $Ca^{2+}$ that strongly influences stability and structure. At low pH, the $Ca^{2+}$ is removed, resulting in reduction in protein stability. α-la gets converted into the molten globule state by removing the $Ca^{2+}$ at low ionic strength (50mM) and neutral pH (Okazaki *et al.* 1994). 'Molten globule' state is a state where apoprotein (calcium-free form) undergoes a change in the three-dimensional structure, while retaining most of its native secondary structure but has no stable tertiary structure (Zhang and Brew 2003).

**2.5.2.3 Bovine serum albumin (BSA)**

BSA is identical to the serum albumin found in the blood. It is synthesized in the liver and through secretary cells gains entry in to milk and accounts for about 5% of the total whey proteins. In the absence of ß-lg, BSA acts as a carrier of retinol (Walstra and Jenness 1984; Haggarty 2003). BSA has one free thiol (Cys 34) and 17 disulphide

linkages, which hold the protein in a structure consisting of nine loops. The molecule is considered to have three major domains, each consisting of two large double loops and a small double loop and assumes an ellipsoidal shape. The N-terminal region is more compact than the C-terminal region (Ng-Kwai-Hang 2003a).

#### 2.5.2.4 Immunoglobulins (Igs)

Igs are the largest and most heterogeneous of the major whey proteins. They are antibodies synthesized in response to stimulation by macromolecular antigens foreign to the animal. Five types of Igs have been classified in bovine milk: IgG, IgA, IgM, IgE and IgD. They account for up to 10% of the whey proteins and exist as monomers or polymers of two kinds of polypeptide chains, light (L) (MW 22,400 Da) and heavy (H) (MW of 50-60,000 Da), each containing about 200 and 450-600 amino acid residues, respectively. The light and heavy chains (two of each) are joined by disulphide linkages to form the basic immunoglobulin structure (Fox and McSweeney 1998).

### 2.6 Binding of vitamin with protein

Milk is an oil-in-water emulsion, hence, possibility exists for addition of 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 milk as the protective coating dissolves 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. These oily vitamin preparations also have a tendency to adhere on the walls of containers and pipelines. Therefore, there is a need for a vitamin complex which will be more soluble and stable. Milk proteins have important functional properties such as the ability to bind hydrophobic molecules, interact with other biopolymers, stabilize emulsions, form gels and to some extent, retard oxidation. Due to these properties, milk proteins are ideal materials for the entrapment and delivery of micronutrients and bioactive compounds (Livney 2010). Binding of vitamin with various milk proteins reduce vitamin degradation during heat treatments, high pressure processing and UV-light induced degradation

process (Liang and Subirade 2010; Saiz-Abajo *et al.* 2013; Semo *et al.* 2007; Zhang *et al.* 2014).

Belatik *et al.* (2012) and Bourassa *et al.* (2013) reported the complexation of retinol and retinoic acid with bovine serum albumin (BSA), α- and β-CN. They analysed retinol and retinoic acid binding sites, binding constant and the effect of retinoid complexation on the stability and conformation of BSA, α- and β-CN using FTIR, UV–visible and fluorescence spectroscopic methods. Structural analysis showed that retinoids bind BSA, α- and β-CN via both hydrophilic and hydrophobic contacts with overall binding constants of $K_{ret\text{-}BSA}$ = 5.3 (±0.8) x$10^6$ $M^{-1}$, $K_{retac\text{-}BSA}$ = 2.3 (±0.4) x 106 $M^{-1}$, $K_{ret\text{-}\alpha\text{-}CN}$ = 1.21 (±0.4) x$10^5$ $M^{-1}$, $K_{ret\text{-}\beta\text{-}CN}$ = 1.11 (±0.5) x $10^5$ $M^{-1}$, $K_{retac\text{-}\alpha\text{-}CN}$ = 6.2 (±0.6) x $10^4$ $M^{-1}$ and $K_{retac\text{-}\beta\text{-}CN}$ = 6.3 (±0.6) x $10^4$ $M^{-1}$. The number of bound retinol molecules per protein (n) was 0.8 (±0.2) for BSA, 1.5 (±0.1) for α-CN and 1.0 (±0.1) for β-CN, while 0.5, 1 and 1 molecule of retinoic acid was bound to BSA, α- and β-CN, respectively. This complexation induced only minor alterations to protein conformation.

Forrest *et al.* (2005) studied the interactions of vitamin $D_3$ with β-lg A and β-CN under various environmental conditions (i.e., pH and ionic strength) using fluorescence and circular dichroism spectroscopic techniques. It was observed that apparent dissociation constant was 0.02 to 0.29 μM and 0.06 to 0.26 μM and apparent mole ratios 0.51-2.04 and 1.16-2.05 (mol of vitamin $D_3$ bound per mole of protein) for β-lg A and β-CN, respectively. According to this study interactions may strongly influence vitamin $D_3$ stability and, hence, bioavailability in processed dairy products. Semo *et al.* (2007) studied the incorporation of vitamin $D_2$ in re-assembled casein micelles and observed that this binding improved the stability of vitamin against UV-light induced degradation without affecting the size and morphology of casein micelles. These complexes can be used as nano vehicles for entrapment, protection and delivery of sensitive hydrophobic nutraceuticals within food products. Diarrassouba *et al.* (2014) also studied the stability of β-lg-vitamin $D_3$ complex at 4°C, upon exposure to UV light and in simulated intestinal conditions using Caco-2 cells. Binding of β-lg to vitamin $D_3$ significantly improved the vitamin stability at 4°C and upon exposure to UV light. Complexation of β-lg to vitamin $D_3$ also improved the crossing of vitamin $D_3$ across monolayer, hence its bioavailability.

Liang and Subirade (2010) studied the binding of folic acid to β-lg and observed it to be bound on the surface of β-lg possibly in the groove between the α-helix and the β-barrel. Complexation of folic acid to β-lg improves its photostability. Zhang *et al.* (2014) also studied the interaction of β-CN with folic acid using fluorescence, absorption spectroscopy and circular dichroism. It was found that folic acid bound to β-CN by hydrophobic contacts with a dissociation constant of ~$10^{-5}$ M. It was also observed that binding to β-CN inhibits the photodecomposition of folic acid. Perez *et al.* (2014a) studied the formation of nano-complexes with folic acid and β-lg and their impact on in-vitro gastro-duodenal proteolysis. Colloidal stability tests ($3 < pH < 10$) revealed that nano-complex formation improved β-lg dissolution near its iso electric point and at low pH values. SDS-PAGE analysis of digesta collected from gastric and duodenal in-vitro digestion of β-lg and its nano-complexes, revealed no marked alterations in the proteolytic susceptibility of β-lg. Theses nano-complexes can be used for the delivery of folic acid in clear beverages with minimal effects to the protein's sensitivity to proteolysis.

Liang *et al.* (2011) studied the complexation of β-lg with α-tocopherol and evaluated its impact on α-tocopherol stability. This interaction did not disrupt the secondary & tertiary structures of β-lg. Complex formation improved the solubility as well as stability of α-tocopherol.

Sáiz-Abajo *et al.* (2013) studied the effect of different processing treatments on stability of β-carotene in re-assembled casein micelles. It was observed that binding of β-carotene in re-assembled casein micelles improves its stability towards sterilisation, pasteurisation, high hydrostatic pressure and baking.

Belatik *et al.* (2012) and Bourassa *et al.* (2013) studied the binding of retinol and retinoic acid with BSA, human serum albumin (HSA), α- and ß-casein. In retinol–BSA complexes, retinol is surrounded by *Arg-209 (2.50 Å = H-bond), Leu-162 (2.38 and 2.42 Å), Phe-150 (1.89 Å), Phe-157 (2.14 and 2.42 Å), Trp-134 (2.52 and 2.39 Å) and Tyr-161 (1.81 Å) with the binding energy of 13.10 kcal/mol. Similarly, retinoic acid is surrounded by *Arg-209 (2.53 Å = H-bond), Ile-165 (2.10 Å), Leu-162 (2.46 and 2.31 Å), Phe-150 (2.16 Å), Phe-157(1.99 Å), Trp-134 (2.55 Å) and Tyr-161 (2.14 Å) with the free binding energy of 12.90 kcal/mol. On the other hand, in retinol–HSA complexes, retinol

is surrounded by Asp-451 (1.83 Å), *Cys-448 (2.79 Å = H-bond), Leu-347 (2.12 Å), Lys-195 (2.00 Å), Trp-214 (2.03 Å), Val-344 (2.14 Å), Val-455 (2.05 and 2.48 Å) and Val-482 (2.14 Å) with the free binding energy of 10.39 kcal/mol. Similarly, retinoic acid is surrounded by Asn-130 (2.09 Å), *Phe-134 (2.49, 2.53 and 2.67 Å = H-bond), Thr-133 (2.31 and 2.17 Å), and Tyr-161 (1.78, 1.92 and 2.22 Å) with the free binding energy of 10.34 kcal/mol. It was evident that several amino acids with hydrophobic and hydrophilic characters are in contact with retinoid in these HSA and BSA complexes. In retinol-*α*-casein complexes, retinol is surrounded by Phe-88, Phe-92, Tyr-89, Tyr-95 and Lys-91 with average binding distances (retinol-amino acid) of 1.7–2.5 Å and a free binding energy of −13.7 kcal/mol, while in retinol-*β*-casein, retinol is located in the vicinity of Tyr-180, Tyr-193, Phe-190, Leu-191, Gly-203, Pro-204, Ile-208 and Val-209 with average binding distances (retinol-amino acid) of 1.5–2.5 Å and a free binding energy of

**Table 2.6: Amino acid involved in protein-retinol/retinoic acid complexes with the free binding energy**

| Complexes | Amino acid residue involved in binding | $\Delta G_{Binding}$ (kcal/mol) |
|---|---|---|
| Retinol-α-casein | Phe-88, Tyr-89, Lys-91, Phe-92, Tyr-95 | −13.7 |
| Retinol-ß-casein | Tyr-180, Phe-190, Leu-191, Tyr-193, Gly-203, Pro-204, Ile-208, Val-209 | −11.1 |
| Retinoic acid-α-casein | Phe-88, Tyr-89*, Lys-91, Phe-92, Tyr-95, Leu-96 | −13.5 |
| Retinol-ß-casein | Tyr-180, Leu-189, Phe-190, Leu-191, Leu-192, Tyr-193, Gly-203, Pro-204, Ile-208, Val-209* | −12.4 |
| Retinol–BSA | Arg*-209, Asn-185, Asp-153, Glu-154, Glu-206, Ile-165, Leu-162, Phe-150, Phe-157, Trp-134, Tyr-161 | −13.10 |
| Retinoic acid–BSA | Arg-209*, Asp-153, Glu-154, Ile-165, Leu-162, Phe-150, Phe-157, Trp-134, Tyr-161 | −12.90 |
| Retinol–HAS | Asp-451, Cys-448*, Leu-198, Leu-347, Leu-481, Lys-195, Ser-454, Trp-214, Tyr-452, Val-343, Val-344, Val-455, Val-482 | −10.39 |
| Retinoic acid–HAS | Ala-126, Arg-117, Asn-130, Leu-115, Leu-182, Lys-137, Met-123, Phe-134*, Phe-165, Thr-133, Tyr-138, Tyr-161 | −10.34 |

*Hydrogen bonding was observed with this residue.

(Belatik *et al.* 2012; Bourassa *et al.* 2013)

−11.1 kcal/mol. In retinoic acid-*α*-casein, acid is surrounded by Phe-88, Phe-92, Tyr-89* (H-bonding), Tyr-95, Lys-91 and Leu-96 with average binding distances (retinol-amino acid) of 1.5–2.7 Å and a free binding energy of −13.5 kcal/mol, while in retinoic acid-*β*-casein, acid is located near Tyr-180, Tyr-193Phe-190, Leu-189, Leu-191, Leu-192, Pro-204, Gly-203, Ile-208 and Val-209* (H-bonding) with average binding distances (retinol-amino acid) of 1.5–2.9 Å and a free binding energy of −12.4 kcal/mol (Table 2.6).

Reports are also available regarding the binding of various tea polyphenols (catechin (C), epicatechin (EC), epigallocatechin (EGC) and epigallocatechin gallate (EGCG)), curcumin and polyunsaturated fatty acid to α-, β-CN, β-lg and re-assembled casein micelles (Esmaili *et al.* 2011; Haratifar and Corredig 2014; Hasni *et al.* 2011; Kanakis *et al.* 2011; Perez *et al.* 2014b; Staszewski *et al.* 2014; Zimet *et al.* 2011).

**2.7 Succinylation of proteins and its effect of protein characteristics**

Studies are available regarding the milk protein-vitamin complex formation but their food application needs solubilisation of these complexes. Succinylation of protein may be a modification which can improve the protein solubility. Succinylation process incorporates additional carboxylic group to free amino and hydroxyl groups of amino acid which further modifies some physico-chemical and biochemical properties of proteins. Modification improved the solubility of casein at its isoelectric point (Schwenke *et al.* 1981). Thompson and Reyes (1980) observed that succinylation resulted in extensive unfolding of protein molecules due to the high negative charge on the protein molecules. It was also observed that succinylation resulted in unaltered amino acid composition with only slight decrease in lysine content of protein. Ser, Thr, Cys, Tyr and His can also be succinylated, although the reaction may be slower or the products are less stable than produced with Lys. Major amino acids involved in the interaction of protein with hydrophobic compounds are Tyr, Phe, Trp, Leu and Val (Belatik *et al.* 2012; Bourassa *et al.* 2013; Forrest *et al.* 2005). Succinylation affect mainly Lys, other amino acid involved in hydrophobic interaction may not be affected. Therefore, modified protein might be able to bind hydrophobic compound to the same extent as unmodified protein. Succinylation also increased the hydrophobicity of milk protein due to

conformational changes which resulted in greater exposure of hydrophobic core. This might be due to increased electrostatic repulsion (Lakkis and Villota, 1992). Choi *et al.* (1981); El-Adawy (2000); Mirmoghtadaie *et al.* (2009); Monteiro and Prakash (1996); Poonampolam *et al.* (1988) and Sheen (1991) also showed the improved oil absorption capacity of various proteins by succinylation. Qasim and Salahuddin (1978) showed that acetylation resulted in exposure of 1 Trp and about 5 Tyr residues as compare to 0.5 Trp and 3 Tyr residues in native ovalbumin. It was observed that exposure of Tyr and Trp residues were only due to electrostatic effects. However, Batra *et al.* (1990) suggested that conformational changes in the secondary structure of ovalbumin were both due the electrostatic effects and stearic hindrance. Hoagland (1966) reported that succinylation of β-CN makes it insensitive to calcium induced coagulation.

### 2.8 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 rational 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 (http://www.a2zproject.org/~a2zorg/pdf/Milk.pdf, 2016).

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 is 3000 µg/day (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 USA, 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).

FSSAI approved the fortification of vitamin A in vegetable oil, milk, vanaspati, *atta, maida* and rice. Vitamin A fortified in vegetable oil, milk, *atta* and *maida* should be in the form of retinyl acetate, retinyl palmitate and retinyl propionate and in case of rice it should be retinyl palmitate. Vitamin A fortified *atta*, *maida* and rice should contain not less than 1500 µg RE/Kg of *atta/maida* or rice. Vitamin A fortified vegetable oil should contain minimum 25 IU/g of oil. Vitamin A fortified toned/double toned/skimmed milk should contain not less than 770 IU/L of milk. Vanaspati is fortified with synthetic vitamin A and it should not be less than 25 IU/g at the time of packing (FSSR 2016).

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, 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.

**2.9 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).

The technology of fortify milk is simple, well established, available and the cost of fortification is low, Vitamin A and D premixes are widely available in India and cost of fortification is around 2-4 paise for fortification/litre of milk (FSSAI 2016).

# CHAPTER –3

# Materials and Methods

The current investigation was carried out for the preparation of milk protein (reassembled casein micelles, native (unmodified) milk protein and modified (succinylated) milk protein) – vitamin A (Vit A) complexes. Native milk protein-Vit A complexes were prepared using sodium caseinate (NaCas), whey protein concentrate (WPC), milk protein concentrate (MPC) and modified milk protein-Vit A complexes were prepared using succinylated sodium caseinate (SNaCas) and succinylated milk protein concentrate (SMPC). Reassembled milk protein-Vit A complexes were prepared using NaCas and SNaCas. Milk protein-Vit A complexes were selected on the basis of vitamin A binding ability of milk proteins and solubility of milk protein-Vit A complexes. Milk protein-Vit A complexes were further characterized for physicochemical properties, stability and *in-vitro* bioavailability of added vitamin A. Stable complexes were used for preparation of fortified milk, which was further analysed for sensory and physicochemical attributes and *in-vitro* bioavailability of added vitamin A.

## 3.1 Materials

### 3.1.1 Protein powders

Sodium caseinate (NaCas) was obtained from Arla Foods Ingredients, Viby J, Denmark. Whey protein concentrate (WPC) was obtained from Davisco Foods

**Table 3.1: Chemical composition of NaCas, WPC and MPC**

| Chemical parameters* | Different protein sources | | |
|---|---|---|---|
| | NaCas | WPC | MPC |
| Protein (%) | 93.50 | 80.0 | 85.15 |
| Lactose (%) | -- | 8.50 | 3.08 |
| Fat (%) | -- | 5.50 | 1.50 |
| Ash (%) | 4.00 | 3.00 | 7.00 |
| Moisture (%) | 6.00 | 4.60 | 5.32 |

*Data from the manufacturer

International, Inc., Minnesota, USA. Milk protein concentrate (MPC) was procured from Mahaan proteins Ltd., New Delhi India. The composition of different protein sources as provided by manufacturers are shown in Table 3.1.

### 3.1.2 Chemical

Fat soluble vitamin A palmitate (170000 IU/g) or free vitamin A (oily form) was procured from Pristine Organics Pvt. Ltd., Bangalore, India.

Folin and Ciocalteu's reagent, sodium carbonate, potassium sulphate, copper sulphate, sodium potassium tartarate, bovine serum albumin (BSA), sodium hydroxide (NaOH), succinic anhydride, lithium hydroxide, acetic acid, hydrindantin, dimethyl sulphoxide (DMSO), L-lysine monohydrochloride, ninhydrin, bromophenol blue, coomassie Brilliant Blue R-250, glycerol, casein standards, glucuronic acid, glucosamine hydrochloride, ammonium sulphate, $\alpha$-amylase from hog pancreas (activity 53.7 units/mg), mucin (type III) from porcine pancreas, pepsin from porcine gastric mucosa (lyophilised powder, activity 2950 units/mg), albumin from bovine serum, 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 (activity 200 units/mg), cholesterol esterase from porcine pancreas (lyophilised, activity 35 units/mg), L-glutamine solution, magnesium chloride, 8-anilino-1-naphthalene-sulfonic acid (ANS), trifluoro acetic acid (TFA) were procured from Sigma Aldrich, St. Louis, MO, USA; Disodium hydrogen phosphate, monosodium dihydrogen phosphate, calcium hydroxide, calcium chloride, sodium chloride, ammonium persulphate (APS) and oxalic acid were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India; N, N, N' N' tetramethylenediamine (TEMED) and glycine were procured from Loba Chemie Pvt. Ltd., Mumbai, India; Tri potassium citrate, isopropyl alcohol (HPLC grade), hydrochloric acid, sulphuric acid and potassium hydroxide were procured from Thermo Fisher Scientific India Pvt. Ltd., Delhi, India; Acetonitrile (HPLC grade), methanol (HPLC grade), chloroform (HPLC grade), hexane (HPLC grade), acetone (HPLC grade), water (HPLC grade) and pyrogallol were procured from Rankem, RFCL Ltd., New Delhi, India; Sodium dodecyl sulphate (SDS) and phenolphthalein was procured from Sisco Research

Laboratories (SRL) Pvt. Ltd., Mumbai, India; Ammonia, iso amyl alcohol, ß-mercaptoethanol, tris and boric acid were procured from Glaxo Laboratories India Pvt. Ltd., Mumbai, India; Ethyl alcohol from Jiangsu Huaxi International Trade Company Ltd., Jiangsu, China; N,N'-methylene-bis-acrylamide, acrylamide sucrose and isopropyl alcohol from s.d. Fine Chem. Ltd. (SDFCL), Mumbai, India; Meito rennet (*Mucor miehei*) (300000 M units/g) from Meito Sangyo Co. Ltd., Nagoya, Japan.

## 3.2 Apparatus and glasswares

All the glassware used in research was of class "A" category. Burette (50 ml), graduated centrifuge tubes (15 ml), measuring cylinders (10, 50, 100, 250, 500 and 1000 ml), volumetric flasks (5, 10, 50, 100, 250, 500 and 1000 ml), petri dishes (200ml and 150ml), conical flask (250ml) and glass test tubes were purchased from Borosil India Ltd., Mumbai, India; Pycnometer, Ostwald's U-tube viscometer, heat stability tubes and clamps were purchased from Laboratory Glass Co. (LABCO), Ambala, India; Whatman filter papers (Whatman no. 1, 4, 41 and 42) from Whatman International Ltd., Kent, England; centrifuge tubes (15 and 50 ml), oak ridge centrifuge tubes, staining box, pasteur pipette, parafilm and gel scoop were procured from Abdos Labtech Pvt. Ltd., New Delhi, India; amicon centrifuge tubes (MW 10KDa), syringe filters (0.22 μm and 0.45 μm, both sterile and non sterile) and disc filters were procured from Millipore India Pvt. Ltd., Bengaluru, India; centrifuge tubes (250 ml) from Kubota Corporation, Tokyo, Japan.

## 3.3 Equipments

1. Weighing balance (BP 221S, Sartorius India Pvt. Ltd., Mumbai, India)
2. pH meter (PICO+, Labindia Instruments Pvt. Ltd., Mumbai, India)
3. Magnetic stirrer (SPINOT MC 02, Tarsons Products Pvt. Ltd., Kolkata, India)
4. Probe sonicator (Sonics and Materials Inc., New Town, USA)
5. Spectrophotometer (UV-2700 230V, SHIMADZU Corporation, Kyoto, Japan)
6. High speed refrigerated centrifuge (KUBOTA-6500, Kubota Corporation, Tokyo, Japan)
7. Lyophilizer (Freezone 6, Labconco Corp., Kansas city, Missouri, USA)

8. Particle size and zeta potential analyzer (Zetasizer Nano ZS, Malvern Instruments Ltd. Malvern, Worcestershire, UK)
9. Scanning electron microscopy (EV018, 18$^{th}$ special edition, Zeiss, Tokyo, Japan)
10. Ion coater (IB-3 Ion coater, Eiko, Tokyo, Japan).
11. High precision water bath (Poly Science, Illinois, USA)
12. Laboratory shaker (Spinix, Vortex shaker, Tarsons Products Pvt. Ltd., Kolkata, India)
13. Auto pipettes (10-100 µl, 20-200 µl, 100-1000µl and 1-10 ml) (Eppendorf India Ltd., Chennai, India)
14. Cream separator (KD-60E, Kamdhenu cream separator, New Delhi, India)
15. Kjeldahl assembly (Gerhardt Analytical systems, Konigswinter, Germany)
16. Hot plate (Advanced Technocracy Inc., Ambala, India)
17. Hot air oven (NSW-143, Narang Scientific Works Pvt. Limited, Delhi, India)
18. BOD Incubator (NSW- 152, Narang Scientific Works Pvt. Limited, Delhi, India)
19. Oilbath (Akash Deep Scientific, New Delhi, India)
20. Hunter lab colorflex colorimeter (Hunter Associates Laboratory Inc., Reston, USA)
21. Refrigerated water bath (Akash Deep Scientific, New Delhi, India)
22. Syringe driven filter unit (33 mm, 0.22 µm pore size) (Millex, Millipore, Billerica, Massachusetts, USA)
23. Hamilton microlitre syringe (25 µl) (Hamilton Company, Nevada, USA)
24. Vacuum filtration assembly (1000 ml) (Schott Duran, Riviera, Wertheim, Germany)
25. Vacuum pressure pump (Millipore India Pvt. Ltd., Bangaluru, India)
26. Ultrasonicator (SONICS, Vibra Cell, Model VCx750, Sonics and Materials Inc., New Town, USA)
27. Electrophoresis unit: 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 wells teflon combs 0.5 mm (2 nos.) and clamps from Sigma Aldrich-Techware, Milwaukee, Wisconsin, USA.

Power supply: Atto (AE-8750 power station 1000XP, ATTO Technology, Inc., New York, USA)

28. HPLC system and accessories: Waters High Pressure Liquid Chromatography 515 (Milford, Massachusetts, USA). It consist 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
29. Fluorescence spectrophotometer (Varian Cary Eclipse, Agilent technologies, Victoria, Australia)
30. Digital water bath (SUB Aqua 18 plus, Thermo Fisher Scientific India Pvt. Ltd., Delhi, India)
31. Pipette controller (Brand Tech Scientific Inc., New Town, USA)
32. Water purifier (Sartorius arium pro, Sartorius India Pvt. Ltd., Mumbai, India)
33. Laboratory shaker (Vortex 3, IKA India Pvt. Ltd., Karnataka, India)
34. Ultrasonicator (SONICS, Vibra Cell, Model VCx750, Sonics and Materials Inc., New Town, USA)

## 3.4 Methods

### 3.4.1 Analysis of protein powders

Protein powders were analysed for protein content by kjeldahl method. The protein content was determined by multiplying the total nitrogen with the factor 6.38. Nitrogen content in each sample was determined according of AOAC method (1970) using Gerhardt automatic nitrogen digestion distillation system.

**Reagents**

0.1 N hydrochloric acid, concentrated sulphuric acid, 4% boric acid, 40% sodium hydroxide, digestion mixture (3.5 g potassium sulphate and 0.105 g copper sulphate were mixed and crushed using pestle and mortar) and mixed indicator (dissolve 0.25 g of methylene blue and 0.375 g of methyl red in 300 ml of 95% ethanol).

**Procedure**

**Digestion:** About 100 mg of the sample was accurately weighed into kjeldahl tubes along with 1g digestion mixture. 10 ml concentrated sulphuric acid was then poured down through the neck of kjeldahl tubes in such a way to wash out any sample particles adhering on the body of the tube. The contents were gently mixed and allowed to stand for 10 min. The digestion tubes were then transferred to the digestion block set at 180°C and samples were digested till white fumes developed. Temperature of the digestion block was then maintained in the range of 410-430°C. Digestion was continued for 2 h, till a clear solution with a greenish tint was obtained. Rack containing the tubes were then removed from the block and allowed to cool for 15 min.

**Distillation:** Distillation was performed on the Vapodest 40 distillation system. Tap water connection to the distillation system was turned ON and after connecting the corresponding tubing to the bottles containing sodium hydroxide solution, boric acid solution (containing few drops of mixed indictor) and distilled water; the Vapodest 40 distillation system was turned ON. A 250 ml conical flask was placed in the distillation system for initial filling of boric acid. The steam inlet tube was then inserted into a digestion tube containing the digested sample. The preset program for automatic distillation was run during which the distilled ammonia was trapped in 50 ml boric acid solution containing mixed indicator. The program set for automatic distillation was as follows:

| **Distillation Programme** | |
|---|---|
| Amount of $H_3BO_3$ | 5s (1s=10ml) |
| Amount of $H_2O$ | 5s (1s=10ml) |
| Amount of NaOH | 6s (1s=10ml) |
| Reaction time | 15 sec |
| Distillation time | 3 min 30 sec |
| Steam power | 90% |
| Suction time | 25 sec |

**Titration:** Boric acid containing trapped ammonia was titrated against standard 0.1 N HCl from burette until the first appearance of violet colour and titre value was recorded. A blank determination was also carried out using sucrose instead of sample.

**Calculation**:

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

Where,

T= Amount of acid required for sample titration (ml)

B=Amount of acid required for blank titration (ml)

N= Normality of acid used for titration (0.1N)

$$Protein\%\ by\ weight = Total\ nitrogen \times 6.38$$

### 3.4.2 Preparation of ß-CN

ß-CN was prepared from both calcium caseinate and skim milk by following the method of Ward and Bastian (1996) and Huppertz *et al.* (2006), respectively. Calcium caseinate was prepared using the method described by GEA Niro (2015). Calcium caseinate (3%) was dissolved in 10mM solution of calcium chloride and stirred for one hour, meanwhile temperature was maintained at 31°C. pH of the solution was adjusted to 6.8, and rennet (300000 M units/g) was added at the rate of 1.5 g/100 L. Curd was evaluated for the proper setting. After proper formation of curd, it was disrupted by magnetic stirring for 2 to 3 min. The aggregated caseinate particles settled at the bottom of the container. The resulting solution was cooled to 4°C and left undisturbed for 48 h for dissociation of ß-CN from caseinate at refrigerated temperature (4-7°C) for 24 h. Samples were centrifuged (5520 g at 4°C for 15 min), supernatant was collected and filtered through Whatman No. 1 filter paper. Filtrate was heated to 45°C in water bath, resulted in precipitation of ß-CN. Sample was again filtered through Whatman No. 1 filter paper. Retentate was collected and lyophilized (−50°C, 6.67 Pa) using a freeze-dryer, powdered and stored in airtight containers.

ß-CN was also prepared by following the method of Huppertz *et al*. (2006). Skim milk was collected from the Experimental Dairy, National Dairy Research Institute, Karnal, Haryana. Skim milk temperature was maintained at 30°C and rennet (300000 M units/g) was added at the rate of 1.5 g/100 L. Sample was maintained at this temperature for 15-30 min for proper setting of the curd. Coagulum was then disrupted and centrifuged at 5000 g for 15 min at 5°C. Supernatant was discarded and curd was redispersed in equal amount of water. Sample was again centrifuged; curd was recollected and redispersed in water similar to the quantity of original milk. Suspension was stored at refrigerated temperature (4-7°C) for 24 h for dissociation of ß-CN from casein micelles. Further, sample was processed by two methods. (a) Suspension was centrifuged at 5000 g for 15 min at 5°C, supernatant was collected, filtered through Whatman No. 1 filter paper. Filtered supernatant was then lyophilized (−50°C, 6.67 Pa) using a freeze-dryer. (b) Calcium chloride was added to the suspension and concentration was maintained as 10mM and further heating of suspension was carried out to 45°C. Suspension was then centrifuged at 5000 g for 15 min at 5°C for complete precipitation of ß-CN. Supernatant was filtered through Whatman No. 1 filter paper. Coagulum was collected and lyophilized (−50°C, 6.67 Pa) using a freeze-dryer.

Both the samples were powdered and stored in airtight containers. Yield of ß-CN was expressed as amount of ß-CN/L of the calcium chloride (3%) solution or skim milk.

#### 3.4.2.1 Purification of ß-CN

Purity of ß-CN was analysed by RP-HPLC method of Jahaniaval *et al.* (2000) with some modifications i.e. centrifugation step and gradient elution sequence was standardized and filtration step was introduced during analysis of the sample.

##### 3.4.2.1.1 Reagents

Solvent A: 0.1% (v/v) Trifluoroacetic acid (TFA) in HPLC grade water

Solvent B: 0.1% (v/v) TFA in a mixture of 60:40 (v/v) acetonitrile (HPLC grade) and HPLC grade water

#### 3.4.2.1.2 Standard preparation

ß-CN standard was dissolved in mixed solvent (solvent A and B - 70:30) and final concentration was adjusted to 25mg/ml of solvent mixture followed by filtration through 0.22 µm syringe filter.

#### 3.4.2.1.3 Sample preparation

25 mg sample of ß-CN was dissolved in one ml of mixed solvent (solvent A and B - 70:30) and centrifuged to 5000 g/30 min followed by filtration through 0.22 µm syringe filter.

#### 3.4.2.1.3 HPLC conditions

20 µL aliquot of the filtrate was analysed using HPLC. Gradient solvent delivery was achieved using Waters Pump Control Module II at a flow rate of 1 ml/min. The separation of different fractions of caseins was achieved with linear gradient elution sequence as described in Table 3.2. The column temperature was maintained at 40°C in column heater chamber. The absorbance of the elute was monitored at 214 nm using Waters 2998 Photodiode Array Detector.

**Table 3.2: Linear gradient elution sequence for caseins**

| Time (min) | Solvent A (%) | Solvent B (%) |
|---|---|---|
| 0 | 55 | 45 |
| 15 | 40 | 60 |
| 20 | 38 | 62 |
| 25 | 37 | 63 |
| 35 | 34 | 66 |
| 45 | 29 | 71 |
| 55 | 26 | 74 |
| 65 | 20 | 80 |
| 70 | 55 | 45 |

### 3.4.3 Protein succinylation

Protein was succinylated following the method of Shilpashree *et al.* (2015a, b) with slight modification i.e. modification in the amount of succinic anhydride per mole of lysine.

### 3.4.3.1 Method for succinylation

Solution of 50 mM lysine (100ml solution was prepared using different protein sources according to the amounts as represented in Table 3.3) was adjusted to pH 8.0 with 4 M NaOH, to this solution known weight of succinic anhydride was then added to obtain a final concentration ranging from 2 to 4 moles/mole of lysine in different protein sources. Sample was stirred for 1 h at 37 °C using magnetic stirrer (solution was constantly monitored for changes in pH, while maintaining the pH at 8.0). Protein was recovered by precipitating the mixture at pH 3.5 – 4.0 with 4 M HCl and centrifugation at 5000 g for 20 min. Protein precipitates were collected, washed by addition of 100 ml of deionized water and stirring for another 1 h, this mixture was again centrifuged. The precipitate was then again washed with 100 ml water and the pH was measured (maintaining the pH in the range of 3.5-4.0 using 4 M HCl). This washing procedure was repeated for another 4 times. Finally, the washed precipitates were resolubilized at pH 7.0 by using 4 M NaOH and then lyophilized (−50°C, 6.67 Pa) using a freeze-dryer, powdered and stored in airtight containers.

**Table 3.3: Required amount of different protein sources for 50 mM of lysine solution**

| Different milk protein sources | Protein content (%) | Lysine content (g/100g of protein) | 50 mM lysine solution (g of protein/100ml of water) |
|---|---|---|---|
| NaCas | 90 | 10.50±0.10 | 8.70 |
| MPC | 85.15 | 10.00±0.17 | 9.14 |

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

### 3.4.3.2 Quantification of succinylation

The extent of succinylation of proteins from different protein sources (NaCas and MPC) was measured according to the method of Friedman *et al.* (1984) with slight modification i.e. the amount of standard (lysine) and sample required for estimation of lysine content was optimized.

#### 3.4.3.2.1 Reagents

**Lithium acetate buffer (4 M):** 168 g of lithium hydroxide was dissolved in 400 ml deionized water and 300 ml of glacial acetic acid was added. The pH was adjusted to 5.2 ± 0.05 with acetic acid or lithium hydroxide as required. Final volume was made upto 1 L and the solution was stored at room temperature.

**Ninhydrin reagent:** Ninhydrin (2 g) and hydrindantin (0.3 g) were dissolved in 75 ml dimethyl sulphoxide (DMSO) solution. After adding 25 ml lithium acetate buffer, the mixture was bubbled with nitrogen for at least 2 min (fresh ninhydrin solution was prepared on each working day).

**Standard preparation:** L–lysine monohydrochloride was used as standard. Calibration curve was drawn in the range of 50 to 500 μM lysine content (Fig 3.1).

#### 3.4.3.2.2 Procedure

5 mg protein sample was dissolved in 5 ml of 0.1 N NaOH. From this, 1 ml sample solution was transferred to a screw-capped test tube along with 1 ml of deionized water to make final volume 2.0 ml. To each tube (both sample and standard) 2.0 ml of freshly prepared ninhydrin solution was added. The tubes were

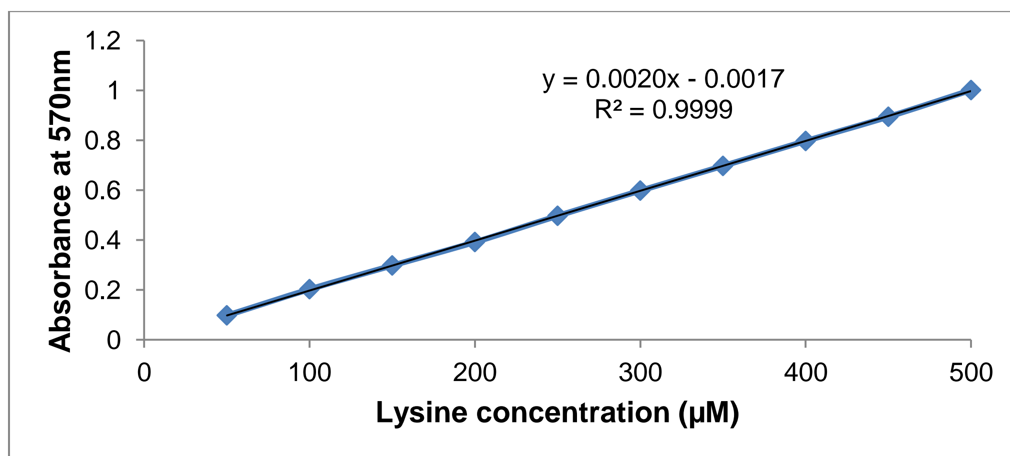



**Fig 3.1: Standard curve for the estimation of lysine content**

placed in a boiling water bath for 15 min. Further, the tubes were immediately cooled in an ice-bath and 6.0 ml of 50% ethanol was added to each tube (total volume 10

ml). Tubes were thoroughly mixed with a vortex mixer for 30 s (presence of any ninhydrin negative and insoluble particles were removed by centrifugation at 2000 g/15 min). Absorbance of the reaction mixture was measured at 570 nm using spectrophotometer.

**Calculation:**

Degree of succinylation was calculated using the following formula:

$$Degree\ of\ succinylation\ (\%) = \frac{A - B}{A} * 100$$

Where, A = µ mole of free amino groups estimated per mg of net protein (native)

B = µ mole of free amino groups estimated per mg of net protein (modified)

### 3.4.4 Preparation of milk protein-Vit A complexes

#### 3.4.4.1 Preparation of vitamin A stock

Oily form of vitamin A palmitate was used for the preparation of vitamin A stock. Vitamin A stock (10 million IU/L i.e. 58.8 mg vitamin A palmitate in 10 ml of absolute alcohol) was prepared by dissolving free vitamin A (oily form) in absolute alcohol and made up the volume with absolute alcohol.

#### 3.4.4.2 Preparation of milk protein–Vit A complexes using stirring

Milk protein-Vit A complexes were prepared by following the method of Diarrassouba *et al.* (2014). Protein solution was prepared by dissolving 5 g protein in 50 ml double distilled water under mild stirring conditions for 2 h at room temperature and then stored overnight at 4°C to allow complete hydration. Complexes were prepared by drop wise addition of vitamin A stock solution (1 ml) to protein solution and further pH was adjusted to 6.7. Volume was made upto 100 ml and stirred continuously for 2 h. Control was prepared by direct addition of vitamin A in double distilled water. Complexes thus formed were lyophilised (−50°C, 6.67 Pa) using freeze-dryer, powdered and stored in airtight containers.

#### 3.4.4.3 Preparation of milk protein–Vit A complexes using sonication

Milk protein-Vit A complexes were prepared by the method described by Ghasemi and Abbasi (2014), with slight modification. Protein (sodium caseinate)

solution was prepared by dissolving 5 g protein in 50 ml double distilled water under mild stirring conditions for 2 h at room temperature and then stored overnight at 4°C to allow complete hydration. pH of the solution was adjusted to 8.0 and sample was stirred for 20 min. Stock solution of vitamin A (1 ml) was added to it and maintained the pH to 8.0 with continuous stirring for 20 min. Ultrasonication was done in water bath maintained at 5°C for 4 min with 25% intensity and further stirring was carried out for 20 min. Final pH of the solution was adjusted to 6.7 and further stirred for 2 h. Control was prepared by direct addition of vitamin A in double distilled water. Complexes thus formed were lyophilised (−50°C, 6.67 Pa) using freeze-dryer, powdered and stored in airtight containers.

#### 3.4.4.4 Preparation of reassembled casein micelle-Vit A complexes

Reassembled casein micelle-Vit A complexes were prepared using the method of Semo *et al.* (2007), with slight modification. On the basis of protein content in the solution, amount of 1 M tri potassium citrate, 0.2 M $K_2HPO_4$ and 0.2 M $CaCl_2$ was optimized.

Protein (NaCas/SNaCas) solution was prepared by dissolving 5 g protein in 40ml double distilled water under mild stirring conditions for 2 h at room temperature (30°C) and then stored overnight at 4°C to allow complete hydration. Vitamin A stock (10 million IU/L) was prepared by dissolving free vitamin A (oily form) in absolute alcohol and made up the volume with absolute alcohol. 2 ml tri-potassium citrate (1M) was added to the protein solution with continuous stirring. After 15 min, 1 ml of vitamin A stock solution was added followed by the addition of 12 ml 0.2 M $K_2HPO_4$ and 10 ml 0.2 M $CaCl_2$. Eight consecutive additions of 1.25 ml 0.2 M $K_2HPO_4$ and 2.5 ml 0.2 M $CaCl_2$ were performed, at 15 min intervals. During this process, sample was stirred using a magnetic stirrer and pH was maintained between 6.7 and 7.0, using 0.1 N HCl or 1 N NaOH. Final pH was adjusted to 6.7 and volume was made up to 100 ml with double distilled water. Sample was further stirred for 2 h and then lyophilized (−50°C, 6.67 Pa) using a freeze-dryer.

**Yield of milk protein-Vit A complexes:**

$$\text{Yield (\%)} = \frac{\text{Weight of milk protein} - \text{Vit A complexes}}{\text{Weight of added protein source}} * 100$$

### 3.4.5 Standardisation of analytical conditions for extraction and estimation of total vitamin A in the sample

#### 3.4.5.1 Sample preparation

1% solution of milk protein-Vit A complexes was prepared (100mg of complex was dissolved in 1 ml absolute alcohol and volume was made up to 10 ml with double distilled water).

#### 3.4.5.1.1 Saponification

Saponification of milk protein-Vit A complexes were done by the method of Kaushik *et al.* (2014a) with slight variation in the amount of different chemicals used for analysis. Sample was treated with 2.5% $NH_4OH$ and 0.1 N HCl prior to saponification to improve the recovery of the fat soluble vitamin A from the complex. Ethanolic pyrogallol was also used as an antioxidant during the saponification treatment. 1ml sample was poured into 15 ml centrifuge tubes. 500 μl of 2.5% $NH_4OH$ (aqueous) was added and vortexed followed by sonication for 10 min. 500 μl of 0.1 N HCl was added and vortexed followed by sonication for 10 min. 500 μl of 60% KOH and 50 μl of 30% alcoholic pyrogallol was then added and again vortexed. Samples were flushed with nitrogen gas, capped and placed in temperature controlled water bath maintained at 70±1°C for 30 min. After 5 min, contents were vortexed and placed back in water bath. Samples were then cooled in refrigerated water bath (4-7°C) for 10 min and vortexed prior to extraction.

#### 3.4.5.1.2 Extraction

Different solvents were evaluated for the extraction of vitamin A from the sample.

1) **Chloroform and methanol as extraction solvent:**

   Chloroform and methanol were used for the extraction of vitamin A from saponified sample (Kazmi *et al.* 2007). The method was modified with regard

to the rate of addition of chloroform and chloroform:methanol mixture and also the speed of centrifugation. 4 ml of chloroform:methanol mixture (1:2) was added to the centrifuge tube (containing saponified sample) and vortexed for 5 min. Further, 1 ml chloroform was added, vortexed and centrifuged at 2236 g for 10 min at 4°C. Two layers were formed after centrifugation and lower layer containing vitamin A was transferred to a glass centrifuge tube using a pasteur pipette. The extract was dried by nitrogen flushing and reconstituted in 1ml mobile phase.

2) **Hexane and absolute alcohol as extraction solvent:**

Hexane and absolute alcohol were used for the extraction of vitamin A from saponified samples (Kaushik *et al.* 2014a). 2 ml of hexane was added to the centrifuge tube containing saponified sample and vortexed for 2-3 min. 200 µl of absolute alcohol was then added to prevent the emulsion formation. Sample tubes were centrifuged at 448 g for 10 min at 4°C. Upper layer was transferred to another glass centrifuge tube and extraction step was repeated twice. Collected hexane layer was dried by nitrogen flushing and reconstituted in 1ml mobile phase.

3) **Hexane, acetone and absolute alcohol as extraction solvent:**

Hexane, acetone and absolute alcohol in the ratio of 2, 1, 1 (v/v/v), respectively were used as extraction solvent (Saiz-Abazo *et al.* 2013). 2 ml of extraction solvent was added to the centrifuge tube containing saponified sample and vortexed for 2-3 min. Sample was centrifuged at 5530 g for 15 min at 4°C and upper layer was collected into glass centrifuge tube. Extract was dried by nitrogen flushing and reconstituted in 1ml mobile phase.

4) **Hexane, absolute alcohol and saturated NaCl as extraction solvent:**

Hexane, absolute alcohol and saturated NaCl was used for the extraction of vitamin A from saponified samples (Chevalier-Lucia *et al.* 2011). 3 ml of absolute alcohol was added to the tube, vortexed for 5 min and then horizontal shaking for 5 min. 1ml hexane was then added and treated similarly. Sample was kept undisturbed for 10 min at 20°C and then 3 ml saturated NaCl solution was added and vortexed. Finally sample was

centrifuged at 1086 g for 10 min at 20°C. Upper layer was collected into glass centrifuge tube and dried by nitrogen flushing. Sample was reconstituted in 1ml mobile phase.

#### 3.4.5.2 HPLC conditions

##### 3.4.5.2.1 Instrumentation

Water 515 HPLC system was used for estimation of vitamin A in milk protein-Vit A complexes and in vitamin A fortified milk. HPLC was equipped with high pressure gradient binary pump system, manual injector, temperature controlled column chamber, 2998 photodiode array (PDA) detector and 2414 refractive index (RI) detector. Waters 2998 PDA detector and 2414 RI detector were connected in series. PDA detector was used as a detector for analysis of vitamin A. Data were collected and analysed using Empower$^2$ software.

##### 3.4.5.2.2 Chromatographic conditions

Analytical column C18 was used for analysis of vitamin A (5 µm, 250 mm x 4.5 mm, 100 Å) with guard column (C18, 100 Å). Operating conditions were: column temperature 40°C, flow rate 1ml/min, injection volume 20 µl, absorption maxima at 325 nm. Two different mobile phases were evaluated for vitamin A analysis on the basis of recovery. Methanol, acetonitrile, water in the ratio of 49.5, 49.5, 1 (v/v/v), respectively and acetonitrile, methanol, chloroform in the ratio of 88, 8, 4 (v/v/v), respectively were evaluated as mobile phase for analysis of vitamin A. Selected mobile phase will be further analysed for the effect of different flow rates on vitamin A analysis. Effect of different injection volumes (20 µl, 100 µl and 200 µl) were further evaluated for sharp peak of the vitamin A.

#### 3.4.5.3 Linearity of calibration curve

Calibration curve was generated by plotting the peak area of standard retinol (vitamin A) versus the theoretical concentration. Calibration curve were run in triplicate and linearity was calculated using linear regression analysis.

### 3.4.6 Standardisation of analytical conditions for extraction and estimation of unbound vitamin A in the sample

Different methods were evaluated for separation of unbound vitamin A from milk protein- Vit A complexes. Vitamin bound to the protein will precipitate with the milk protein and unbound vitamin will remain in the solution, hence, different methods of protein precipitation were evaluated for analyzing unbound vitamin A. Centrifugation and ultrafiltration techniques were utilised for estimation of unbound vitamin A. Protein was precipitated by isoelectric precipitation and also by ammonium sulphate precipitation. TCA precipitation and careez precipitation disrupts the structure of protein or may affect the binding of vitamin A to milk protein, therefore were not used for the estimation of unbound vitamin A in the sample.

1% solution of milk protein-Vit A complexes were prepared (100mg of complex was dissolved in 1 ml absolute alcohol and volume was made up to 10 ml with double distilled water).

1) **Centrifugation method**

   Studies are available regarding the interaction of hydrophobic compounds to milk proteins. Different workers reported the use of different centrifugation speeds for separation of unbound compounds (Benzaria *et al.* 2013; Chevalier-Lucia et al. 2011; Elzoghby et al. 2013; Haratifar and Corredig 2014; Menendez-Aguirre *et al.* 2014; Somchue *et al.* 2009). Menendez-Aguirre *et al.* (2014) used highest centrifugation speed (25000 g) for separation of free α-tocopherol from protein bound α-tocopherol. Milk protein-Vit A complexes (1% solution) was prepared and analysed for vitamin A content. Sample was centrifuged at 25000 g for 30 min at 20°C to separate unbound vitamin A from the complexes. Vitamin A content was analysed in supernatant after centrifugation.

$$Binding\ ability = \frac{Total\ vitamin\ A\ in\ the\ sample - Vitamin\ A\ content\ in\ supernatant}{Total\ vitamin\ A\ in\ sample} * 100$$

**2) Ultrafiltration (UF) method**

UF was carried out according to the method of Shilpashree *et al.* (2016) with slight modification in the speed of centrifugation. Milk protein-Vit A complexes (1% solution) were prepared and analysed for vitamin A content. Sample was centrifuged at 18000 g at 20°C for 30 min instead of 12000 g to avoid clogging of UF membrane during UF. Supernatant which contained soluble vitamin A and protein was carefully decanted and filtered through Whatman No. 1 filter paper. The filtered supernatant was then passed through an Amicon UF membrane centrifuge tubes (MW cut off 10 KDa). UF centrifuge tubes were centrifuged at 2236 g for 30 min at room temperature. Vitamin A content was analysed in the permeate.

$$Binding\ ability = \frac{Total\ vitamin\ A\ in\ sample - Vitamin\ A\ content\ in\ permeate}{Total\ vitamin\ A\ in\ sample} * 100$$

**3) Isoelectric precipitation**

Isoelectric precipitation of complexes was carried out according to the method of Zimet *et al.* (2011). 1% solution of milk protein-Vit A complexes was prepared and analysed for vitamin A content. Milk protein-Vit A complexes (1% solution) were isoelectrically precipitated by adjusting the pH to 4.6 with 0.1 N HCl. Sample was centrifuged at 3000 g for 15 min at 20°C to separate the unbound vitamin A from protein bound vitamin A. Vitamin A content was analysed in the supernatant after centrifugation of the sample.

$$Binding\ ability = \frac{Vitamin\ A\ content\ in\ precipitate}{Total\ vitamin\ A\ in\ sample} * 100$$

**4) Ammonium sulphate precipitation**

Ammonium sulphate precipitation of milk protein-Vit A complexes was carried out by following the method of Blayo *et al.* (2014) with modification. Sample was centrifuged at 18000 g instead of 12061 g. 1% solution of milk protein-Vit A complexes was prepared and analysed for vitamin A content. Complete precipitation of protein was achieved at 2 M ammonium sulphate concentration. One ml of sample (1% solution) was added with 9 ml of

phosphate buffer (10 mM, pH 6.6) containing adequate amount of ammonium sulphate to obtain a final concentration of 2 M ammonium sulphate. Sample was kept at 20°C for 2 h away from light; then centrifuged at 18000 g for 30 min at 20°C for complete precipitation of milk protein. Supernatant and precipitate both were analysed for the vitamin A content.

$$Binding\ ability = \frac{Vitamin\ A\ content\ in\ precipitate}{Total\ vitamin\ A\ in\ sample} * 100$$

$$Binding\ ability = \frac{Total\ vitamin\ A\ in\ sample - Vitamin\ A\ content\ in\ supernatant}{Total\ vitamin\ A\ in\ sample} * 100$$

### 3.4.7 Selection of milk protein-Vit A complexes

#### 3.4.7.1 Solubility

Solubility of protein–Vit A complexes was evaluated using the Method of Lawal *et al.* (2007) with slight modification i.e. protein was estimated by Lowry method instead of Kjeldahl.

**Procedure:**

1.0 g of sample was dispersed in 100 ml phosphate buffer (0.05 M, pH 7.0) and the solution was adjusted to pH 7.0 using 0.1N NaOH. The solution was mixed for 1 h at 30°C using magnetic stirrer followed by centrifugation at 18000 g for 20 min at 4°C. The supernatant was filtered through Whatman No. 1 filter paper and protein content was determined using the method of Lowry *et al.* (1951). Determinations were carried out in triplicate and the percentage of solubility was evaluated as following.

**Calculation:**

$$Solubility\ of\ protein\ (\%) = \frac{Amount\ of\ protein\ content\ in\ supernatant}{Amount\ of\ protein\ in\ the\ sample} * 100$$

$$Solubility\ of\ vitamin\ (\%) = \frac{Amount\ of\ vitamin\ content\ in\ supernatant}{Amount\ of\ vitamin\ in\ the\ sample} * 100$$

#### 3.4.7.2 Binding ability

The ability of milk protein to bind vitamin A in both solution and dry form was analysed as follows:

$$Binding\ ability = \frac{Free\ vitamin\ A}{Total\ vitamin\ A} * 100$$

### 3.4.8 Physicochemical characterization of milk protein–Vit A complexes

**Sample preparation**

1% solution of milk protein-Vit A complexes and free vitamin A (oily form) was prepared (100mg of sample was dissolved in 1 ml absolute alcohol and volume was made up to 10 ml with double distilled water).

#### 3.4.8.1 Vitamin A content in milk protein-Vit A complexes

10 mg of milk protein-Vit A complex was dissolved in 100 µl absolute alcohol, vortexed for 5 min and then addition of 900 µl of double distilled water. 1ml sample was transferred to the 15 ml centrifuge tube. 500 µl of 2.5% $NH_4OH$ (aqueous) was added and vortexed followed by sonication for 10 min. 500 µl of 0.1 N HCl was added and vortexed followed by sonication for 10 min. 500 µl of 60% KOH and 50 µl of 30% alcoholic pyrogallol was then added and again vortexed. Samples were flushed with nitrogen gas, capped and placed in temperature controlled water bath maintained at 70±1°C for 30 min. Samples were vortexed at a time interval of 5 min. Samples were then cooled in refrigerated water bath for 10 min and vortexed prior to extraction. 4 ml of chloroform:methanol mixture (1:2) was added and vortexed for 5 min. Further, 1 ml chloroform was added, vortexed and centrifuged at 2236 g for 10 min at 4°C. Two layers were formed after centrifugation and the lower layer containing vitamin A was transferred to a glass centrifuge tube using a pasteur pipette. Extract was dried under nitrogen and reconstituted in 1ml mobile phase.

Sample was further analysed by Water 515 HPLC system using analytical column C18 (5 µm, 250 mm x 4.5 mm, 100 Å) with guard column (C18, 100 Å). The mobile phase consisted of methanol: acetonitrile: water (49.5: 49.5: 1) was pumped at a flow rate of 1.0 ml/min. Column temperature was maintained at 40°C using a

column heater chamber. The sample volume injected was 20 µl and a PDA detector at 325 nm was used to monitor the eluate.

### 3.4.8.2 Protein content in milk protein-Vit A complexes

#### 3.4.8.2.1 Kjeldahl method:

The protein content was determined as described in the section 3.4.1.

#### 3.4.8.2.2 Folin–phenol method:

Protein content was determined by Folin-phenol method as described by Lowry *et al.* (1951)

**Reagents:**

**Reagent A:** 1 g copper sulphate was dissolved in 100 ml distilled water.

**Reagent B:** 2 g of sodium potassium tartrate was dissolved in 100 ml distilled water.

**Reagent C:** Sodium carbonate 2% (w/v) in 0.1 N NaOH (21.2 g of sodium carbonate and 4 g NaOH were dissolved in 1 L of distilled water).

**Alkaline reagent:** 0.5 ml of reagent A, 0.5 ml of reagent B and 100 ml of reagent C were mixed immediately before use.

**Folin's reagent (1 N):** Folin's reagent (2 N, commercially available) was diluted with an equal volume of water immediately before use.

**Standard protein:** Bovine serum albumin (BSA) was used as a standard protein and different concentrations i.e.100 to 1000 µg/ml were made in phosphate buffer (20mM, pH 7.0).

**Procedure:**

Sample preparation: Samples were prepared by dissolving 1.0 g of milk protein-Vit A complexes in 100 ml of water (1% solution) and further samples were diluted 10 times for analysis of protein content. Different reagents were added to different standard and sample solutions as given in Table 3.4.

After incubation, the colour was measured at 660 nm using spectrophotometer. The standard curve was constructed by plotting a graph of standard concentrations versus absorbance as shown in Fig 3.2. Finally the protein content in the sample was determined by interpolating the standard curve.

**Table 3.4 Standard procedure for the estimation of protein**

| Reagents | Blank (Distilled water) | Standards | Sample |
|---|---|---|---|
| | | **S1 to S10** | **S1 to S3** |
| Standard/Sample (ml) | 0.5 | 0.5 | 0.5 |
| Alkaline reagent (ml) | 5 | 5 | 5 |
| Mix and incubate at room temperature (30°C) for 10min | | | |
| Folin's reagent (ml) | 0.5 | 0.5 | 0.5 |
| Vortex each tube immediately after reagent is added and incubate at room temperature (30°C) for 30min | | | |

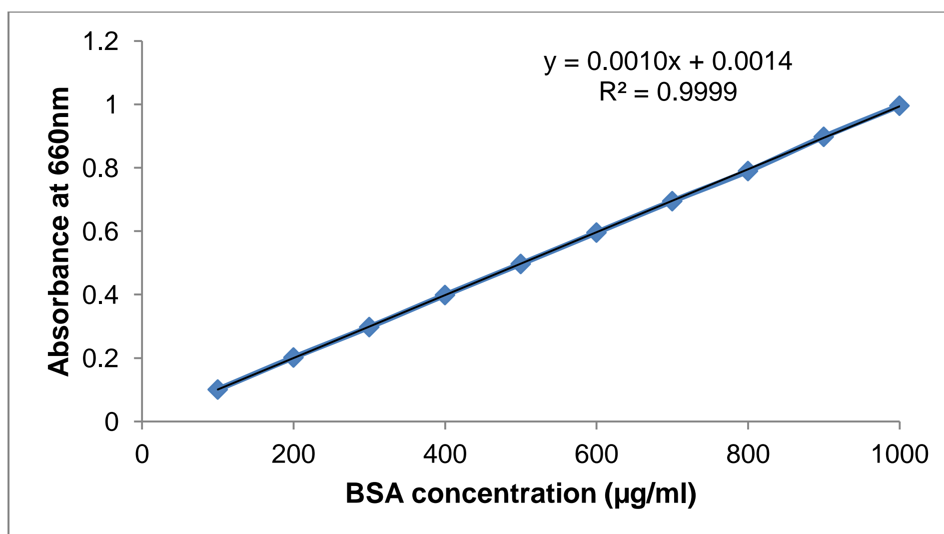



**Fig 3.2: Standard curve for the estimation of protein content**

**Calculation:**

Protein content (µg/ml) = Concentration of protein from standard curve * dilution factor

### 3.4.8.3 Turbidity analysis to elucidate solubility

Turbidity analysis was carried out by measuring the transmittance at 450 nm and turbidity was expressed as:

$$\text{Turbidity} = 100 - \%\text{Transmittance}$$

Milk protein-Vit A complexes and free vitamin A (oily form) were dissolved in double distilled water to maintain vitamin A concentration 10 lakh IU/L. Effect of milk protein concentration on turbidity was eliminated by measuring the turbidity of milk protein solutions which contained similar protein concentration as present in the complexes.

#### 3.4.8.4 Particle size and zeta potential analysis

Particle size and zeta potential of protein samples were determined at 25°C by dynamic light scattering and laser doppler micro-electrophoresis technique, respectively. Particle size analysis was based on the time dependent fluctuation of scattering of laser light by the particles undergoing brownian motion and zeta potential was based on the direction and velocity of particles under the influence of known electric field. Samples were prepared by dissolving 1.0% (w/v) protein in 0.05 M phosphate buffer (pH 7.0) and further, samples were diluted 50 times for measurement of particle size and zeta potential. Three measurements were carried out for each protein sample.

#### 3.4.8.5 Microstructural analysis by scanning electron microscopy

The microstructure of milk protein-Vit A complexes were examined by scanning electron microscopy (SEM). Lyophilized powder samples were sprayed on double adhesive tape mounted on aluminum stub. Mounted samples were coated with gold (20 nm thickness) on ion coater at 0.05-0.07 torr for 4 min maintaining the ion current at 6 mA. Samples were finally examined by SEM at an acceleration voltage of 15 KV under high vacuum ($9.0 \times 10^{-5}$ torr) and micrographs were recorded.

#### 3.4.8.6 Evaluation of the effect of vitamin binding on the mobility of protein

SDS is a negatively charged surfactant. It is used to disrupt non-covalent bonds through its ability to absorb on hydrophobic and positively charged sites on proteins. Most proteins bind similar amounts of SDS on mass basis. The SDS-protein complexes are then separated on the basis of molecular size of the protein. Samples are reduced by treatment with 2-mercaptoethanol to disrupt intra-and inter-

molecular 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)): 29.2% acrylamide and 0.8% 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, 10 ml ß-mercaptoethanol and 20 ml 10% SDS were mixed together to prepare sample buffer
9. 5X Electrode buffer (25 mM Tris-HCl (pH 8.3 (adjusted with 6 M HCl)), 192 mM glycine, 0.1% SDS): 15 g Tris, 72 g glycine and 5 g SDS was dissolved in distilled water and volume was made upto 1 litre. Buffer was diluted five times before use.
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**: 2.5 mg protein of each sample was dissolved per ml sample buffer containing 20 µl mercaptoethanol/ml. Samples were then heated for 4 min in boiling water bath and then cooled to room temperature.

    15 µl of marker was dissolved in 15 µl of sample buffer and 10 µl of standard was dissolved in 40 µl of sample buffer. Standard was prepared from HPLC standards of α-, ß- and κ-CN. The prepared standard contained 4.6 mg/ml α-CN, 3.84 mg/ml ß-CN and 1.22 mg/ml κ-CN.

13. **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 |

14. **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 |

Note: The working solutions were filtered and stored at 6°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 the help of plastic gaskets and clamps. The required quantity of the gel ingredients were measured except APS and TEMED into a 100 ml Buchner flask and contents were degassed under vacuum, with stirring for 15 min. APS and TEMED were added gently with swirling after each addition. 3.30 ml of resolving gel solution was poured in between the assembled electrophoresis glass plates. 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 min 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 h. The combs were removed and sample wells were overlaid with enough water to fill completely. Clamps and gaskets were removed from the plates. The electrode buffer was poured in the container upto ½ level and then glass assembly was placed inside, avoiding the 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 | : | 0.5-1.1 h |

**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 h after which the gel was transferred to destaining solution. Destaining was done overnight, till the bands appear and background became clear. The gel was then transferred to distilled water and photographs of the gel were captured.

### 3.4.8.7 Evaluation of the effect of vitamin binding on Tryptophan fluorescence intensities

#### 3.4.8.7.1 Intrinsic tryptophan intensity

Intrinsic tryptophan intensity was measured according to the method of Zhou *et al.* (2012) to determine the intrinsic tryptophan intensities of milk protein–Vit A complexes.

**Procedure:**

0.01% protein solution of each sample in phosphate buffer (10mM, pH 7.0) was prepared and evaluated for tryptophan intensity over the emission wavelength

ranging from 300 to 500 nm at a fixed excitation wavelength of 280 nm with slit width 5 nm.

#### 3.4.8.7.2 Hydrophobicity

Hydrophobicity of milk protein–Vit A complexes was determined using the method of Yuksel *et al.* (2010).

**Procedure:**

1.0% protein solution of each sample was prepared in phosphate buffer (10mM, pH 7.0). The fluorescent probe used was 8-anilino-1-naphthalene sulfonic acid (ANS). Relative fluorescence of the samples were measured by using fluorescence spectrophotometer at excitation wavelength 390 nm, emission wavelength 480 nm with slit width 5 nm. 10 mM ANS (30 mg of ANS was dissolved in 300 μl of 1N NaOH and volume was made upto 10 ml with distilled water) concentration was prepared and used for ANS titration. Final concentration of ANS in the protein solution was between 0 and 140 μM during the titration. The main aim of this parameter was to reach the maximum fluorescence that showed a saturated fluorescent marker (ANS) binding. Before ANS titration, fluorescence of the samples was measured as blank. Kinetic data were also evaluated from ANS titration curves according to the method of Guo *et al.* (1996).

#### 3.4.8.8 Evaluation of the effect of vitamin binding on the elution characteristic of protein by RP-HPLC

RP-HPLC was carried out as described earlier in section 3.4.2.1 to determine the elution profile of the milk protein-Vit A complexes.

**Standard preparation:** Standards were dissolved in HPLC grade water, the final concentration of standards was adjusted as follows - β-CN 3.84mg/ml, α-CN 4.6 mg/ml and κ-CN 1.22 mg/ml of HPLC grade water. Mix standard was also prepared by dissolving the same concentrations of proteins (as discussed above) in 1 ml of HPLC grade water, followed by filtration through 0.22μm syringe filter.

**Sample preparation:** 1.0% protein solution of each sample was prepared in HPLC grade water and centrifuged to 5000 g/30 min followed by filtration through 0.22μm syringe filter.

**HPLC conditions**

20 µl aliquot of the filtrate was analysed using HPLC with flow rate of 1 ml/min. The column temperature was maintained at 40°C in column heater chamber. The absorbance of eluent was monitored at 214 nm using Waters 2998 Photodiode Array Detector.

### 3.4.9 Evaluation of stability of vitamin A in milk protein–Vit A complexes:

Stability of vitamin A in milk protein-Vit A complexes was evaluated in comparison to the free vitamin A (oily form). Selected complexes and free vitamin A (oily form) were stored in microcentrifuge tubes (1.5 ml, virgin polypropylene) and aluminium laminate (Polyethylene terephthalate or nylon/Aluminium foil/polypropylene, thickness 0.11 mm) pouches. 10 mg sample was stored in microcentrifuge tube and 100 mg sample was stored in aluminium laminate pouches at three different temperatures -20°C, 4°C and 37°C and evaluated for vitamin A content at a time interval of one month upto 6 months.

$$Stability\ (\%) = \frac{Vitamin\ A\ content\ after\ storage}{Vitamin\ A\ content\ before\ storage} * 100$$

### 3.4.10 Evaluation of the effect of pH on stability of milk protein–Vit A complexes:

Selected milk protein-Vit A complexes were suspended in distilled water and pH was adjusted to 3.0, 5.0 and 7.0 using 0.1 N NaOH and 0.1 N HCl and made up the volume to 100 ml. Samples were stored at 4°C for 24 h followed by centrifugation at 2236 g for 30 min. Vitamin A and protein content were analysed in samples before centrifugation and in supernatant after centrifugation.

$$Solubility\ (\%) of\ vitamin\ A = \frac{Vitamin\ A\ in\ supernatant}{Vitamin\ A\ in\ sample} * 100$$

$$Solubility\ (\%) of\ protein = \frac{Protein\ in\ supernatant}{Protein\ in\ sample} * 100$$

### 3.4.11 *In-vitro* bioavailability of vitamin A from milk protein-Vit A complexes and free vitamin A (oily form):

Bioavailability of vitamin A from milk protein–Vit A complexes and free vitamin A (oily form) was evaluated using the method of Gupta *et al.* (2015). Composition and concentration of the various synthetic juices of the *in vitro* digestion are presented in Table 3.5.

Vitamin A concentration in the double distilled water was maintained at 10000 IU/L by dissolving milk protein-Vit A complexes and free vitamin A (oily form). Approximately, 5 ml of each sample solutions was transferred to a flask and saliva solution (1.92 ml, pH 6.5) was added after which the samples were incubated in a shaking water bath at 37°C, 95 rpm for 5 min. After incubation gastric juice (2.89 ml) was added, pH was adjusted to 1.1 with HCl and the solution was incubated for 2 h at 37°C. Freshly prepared duodenal juice (5.35 ml) and bile solution (1.92 ml) were then added to the solution after neutralization of the pH (7.8). It was then incubated at 37°C for 3 h. Further, volume was made upto 20 ml.

According to Veda *et al.* (2006), fat soluble compounds are converted into micellar fraction during *in-vitro* digestion and these micellar fractions are available for absorption. Micellar fraction was separated from digesta by following the method of Veda *et al.* (2006), with slight modification. Digested samples were centrifuged at 18000 g for 30 min instead of 5000 g for 20 min at 4°C for precipitation of non-micellarised fraction. Further, samples were filtered through 0.22 μm syringe filters instead of 0.65 μm syringe filters to remove microcrystalline non-micellarised vitamin A. Vitamin A content was estimated in the micellar fraction.

$$In-vitro\ \text{bioavailability} = \frac{\text{Vitamin A content in micellar fraction}}{\text{Vitamin A content in the sample}} * 100$$

### 3.4.12 Preparation of vitamin A fortified milk:

#### 3.4.12.1 Collection of milk samples

Fresh cow and buffalo milk were collected from the herd of cows and buffaloes maintained in the Livestock Research Centre, National Dairy Research Institute, Karnal, India after the morning milking. Cream was then separated with the

**Table 3.5: Composition and concentration of the various synthetic juices utilized during *in vitro* digestion**

| Solution↓ | Saliva | Gastric Juice | Duodenal juice | Bile |
|---|---|---|---|---|
| Inorganic solution | 1 ml KCl (89.6 g/L)<br>1 ml KSCN (20 g/L)<br>1 ml $NaH_2PO_4$ (88.8 g/L)<br>1 ml $Na_2HPO_4$ (57 g/L)<br>0.17 ml NaCl (175.3 g/L)<br>0.18 ml NaOH (40 g/L) | 1.57 ml NaCl (175.3 mg/L)<br>0.3 ml $NaH_2PO_4$ (88.8 mg/L)<br>0.92 ml KCl (89.6 g/L)<br>0.18 ml $CaCl_2$. 2 $H_2O$ (22.2 mg/L)<br>1.0 ml $NH_4Cl$ (30.6 g/L)<br>0.83 ml HCl (37%) | 4 ml NaCl (175.3 mg/L)<br>1 ml $NaHCO_3$ (84.7 mg/L)<br>1 ml $KH_2PO_4$ (8 g/L)<br>0.63 ml KCl (89.6 g/L)<br>1 ml $MgCl_2$ (5 mg/L)<br>18.0 μl HCl (37%) | 3.0 ml NaCl (175.3 g/L)<br>6.83 ml $NaHCO_3$ (84.7 g/L)<br>0.42 ml KCl (89.6 g/L)<br>20.0 μl HCl (37%) |
| Organic solution | 0.8 ml urea (25 g/L) | 1 ml glucose (65 g/L)<br>1 ml glucuronic acid (2 g/L)<br>0.34 ml urea (25 g/L)<br>1 ml glucosamine hydrochloride (33 g/L) | 0.4 ml urea (25 g/L) | 1 ml urea (25 g/L) |
| Add to mixture organic+ inorganic solution | 14.5 mg α-amylase<br>1.5 mg uric acid<br>5.0 mg mucin | 0.1 g BSA<br>0.1 g pepsin<br>0.3 g mucin | 0.9 ml $CaCl_2$. 2 $H_2O$ (22.2 g/L)<br>0.1 g BSA<br>0.3 g pancreatin<br>Pancreatic lipase (1 units)<br>1.25 μg colipase<br>Cholesterol esterase (1 units)<br>5.0 μl phospholipase $A_2$<br>1.99 mg sodium taurocholate | 1 ml $CaCl_2$. 2 $H_2O$ (22.2 g/L)<br>0.18 g BSA<br>0.6 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 50 ml with distilled water | | | | |

help of cream separator. Fat and SNF of milk were adjusted to 3% and 8.5%, respectively with cream, skim milk and SMP using Pearson's square method.

### 3.4.12.2 Analysis of milk

#### 3. 4.12.2.1 Fat

The fat content of milk was determined by the Gerber method as described in IS: 1224: 18 (Part 1) (1977). 10 ml Gerber sulphuric acid was transferred into a Gerber butyrometer using an automatic measure. 10.75 ml of well mixed milk sample was pipetted out slowly into the butyrometer along the sides. 1 ml of iso amyl alcohol was then added with an automatic measure. The butyrometer was closed firmly with the stopper and shaken carefully to mix the contents and centrifuged at 1100 rpm for 4 min. Butyrometer was then placed in water bath maintained at 65°C for 5 min. The difference between the scale readings corresponding to the lowest point of fat meniscus and the surface of separation of fat and acid gave the % fat content in milk.

#### 3. 4.12.2.2 Solid-not-fat (SNF)

The SNF content in milk was determined using the method as described in IS: 10083 (1992). Lactometer reading was recorded at 27°C

$$\mathrm{SNF}\ (\%\ \mathrm{weight}) = 0.25\ (\mathrm{CLR} + \mathrm{F}) + 0.44$$

Where,

CLR= Corrected Lactometer reading (or Lactometer reading at 27°C)

F= Fat% determined by Gerber method

### 3.4.12.3 Procedure

Toned milk was transferred in clean and dried container and calculated amounts of selected milk protein-Vit A complexes and free vitamin A (oily form) were weighed separately and added to fortify milk. Milk protein-Vit A complexes were suspended in distilled water, however, free vitamin A (oily form) was initially suspended in alcohol and further dissolved in water for ensuring the solubility of free vitamin A (oily form) in milk. Addition of fortificants, colour, flavour and sugar were accompanied by thorough mixing for 10 min for complete dissolution of fortificants. The milk samples were then pasteurised at 63ºC for 30 min followed by cooling to 4ºC in refrigerated water bath. Sensory analysis of milk samples was conducted after keeping the pasteurised milk

samples for 2 h under refrigerated conditions (4-7ºC). Figure 3.3 illustrates the major steps involved in fortification of liquid milk.

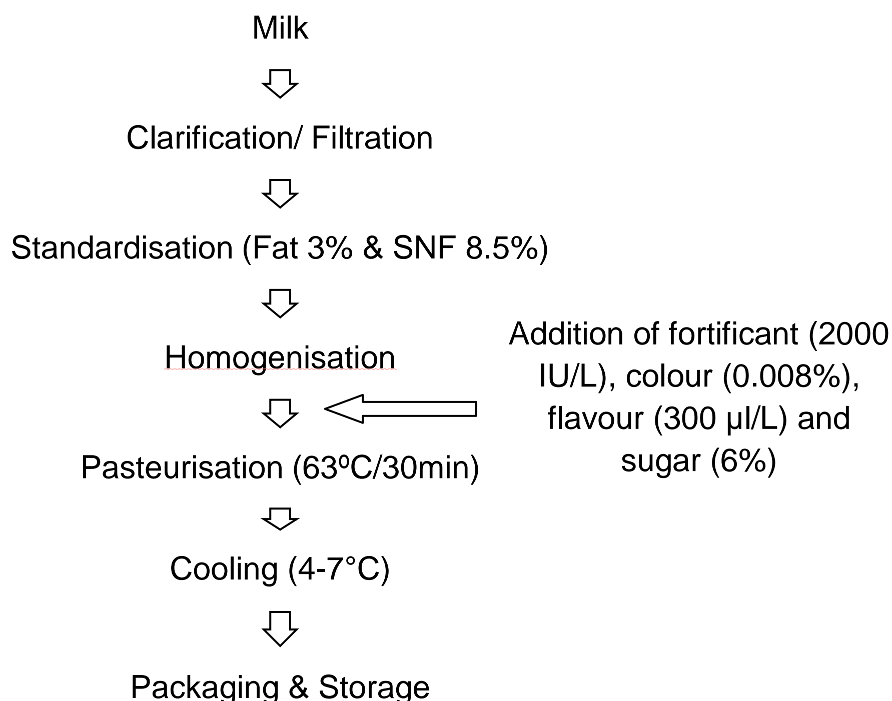



**Figure 3.3: Flow diagram for preparation of vitamin A fortified pasteurized milk**

### 3.4.12.4 Addition of vitamin A fortificants

Vitamin A content was estimated in selected milk protein-Vit A complexes and free vitamin A (oily form). These fortificants were used for the fortification of toned milk. Milk was fortified with fortificants at the rate of 2000 IU per litre. One International unit (IU) of vitamin A is equivalent to 0.3 µg of vitamin A or retinol. Stock solutions (2000 IU/ml) were prepared by dissolving the different amount of fortificants on the basis of their vitamin content. Calculated volume of stock solutions was added to milk to achieve the desired levels of fortification.

### 3.4.12 Evaluation of sensory and physicochemical attributes of milk protein-Vit A complexes and free vitamin A (oily form) fortified milk

### 3.4.13.1 Sensory evaluation

Sensory evaluation was carried out by a panel of ten trained judges who graded control (unfortified) and vitamin A fortified milk (containing selected complexes and free

vitamin A (oily form)) for any change in colour and appearance, odour, taste and mouthfeel. Composite score card for sensory analysis of pasteurised milk as developed by BIS (IS: 7768, 1975) was used. The composite sensory score card is designated as Annexure I in the thesis. All sensory assessments took place in 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.

#### 3.4.13.2 Physicochemical properties

Milk samples were evaluated for the following physicochemical properties.

##### 3.4.13.2.1 pH

pH of the milk samples were determined electrometrically with pH meter by the method as 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.

##### 3.4.13.2.2 Titratable acidity

**Phenolphthalein indicator solution**

1 g of phenolphthalein was weighed and transferred to a 100 ml volumetric flask containing about 50 ml of 95% ethanol. The flask was stoppered and shaken vigorously for few minutes. Solution was then neutralized with 0.1 N NaOH. 20 ml ethanol was then further 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 and standardized against 0.1 N oxalic acid (primary standard). It was then stored in an amber coloured glass bottle.

Normality of NaOH ($N_2$)= $N_1 * V_1 / V_2$

Where,

$N_1$ = Normality of oxalic acid (0.1N)

$V_1$ = Volume of oxalic acid

$N_2$ = Normality of NaOH

$V_2$ = Volume of NaOH used

**Procedure**

Titratable acidity of the milk samples were estimated according to the method as described in IS: SP:18, part XI (1981). 10 ml milk sample was pipetted in a conical flask and 2-3 drops of phenolphthalein indicator was added to it. Sample was then titrated with 0.1 N NaOH to a persistent pink colour. Acidity was recorded as % lactic acid by volume.

$$\text{Acidity (\% lactic acid)} = \frac{9 * N * A}{V}$$

Where,

N = Normality of 0.1 N NaOH

A = Volume of 0.1 N NaOH used

V = Volume of the milk sample

**3.4.13.2.3 Curd tension**

Curd tension of control (unfortified) and vitamin A fortified milk (containing selected complexes and free vitamin A (oily form)) was determined as per the method described by Kaushik *et al.* (2015a).

**Procedure:**

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±1$^{o}$C. The curd tension knife was then placed in the beaker and 0.5 ml of 0.015% (w/v) Meito rennet (3000000 M units/g) was added. Milk was stirred immediately and was placed in an incubator maintained at 30±1$^{o}$C. 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.

#### 3.4.13.2.4 Viscosity

Absolute viscosity of control (unfortified) and vitamin A fortified milk (containing selected complexes and free vitamin A (oily form)) was measured at 27°C with an Ostwald viscometer as described by Lewis (1996). The density of samples at 27°C was analysed using pycnometer. The specific gravity, relative viscosity, absolute viscosity and kinematic viscosity were calculated according to the following formula.

$$Specific\ gravity = \frac{wt.of\ pycnometer\ fill\ with\ milk - wt.of\ empty\ pycnometer}{wt.of\ pycnometer\ fill\ with\ water - wt.of\ empty\ pycnometer}$$

$$Relative\ viscosity = specific\ gravity * \frac{time\ taken\ by\ milk}{time\ taken\ by\ water}$$

$$Absolute\ viscosity = Relative\ viscosity * viscosity\ of\ water\ at\ 27\ °C$$

$$Kinematic\ viscosity = \frac{Absolute\ viscosity}{density\ of\ water\ at\ 27\ °C}$$

#### 3.4.13.2.5 Rennet coagulation time:

Rennet coagulation time (RCT) of control (unfortified) and vitamin A fortified milk (containing selected complexes and free vitamin A (oily form)) was determined according to the method described by Kaushik *et al.* (2015a).

**Procedure:**

10 ml milk was transferred to a test tube and 1 ml of 0.2% rennet (3000000 M units/g) 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.

#### 3.4.13.2.6 Alcohol stability

Alcohol stability of control (unfortified) and vitamin A fortified milk (containing selected complexes and free vitamin A (oily form)) 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 w/w or 75% by w/v; 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.

**3.4.13.2.7 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 Kaushik *et al*. (2015b).

**3.4.13.2.7.1 Adjustment of pH**

Each control (unfortified) and vitamin A fortified milk (containing selected complexes and free vitamin A (oily form)) sample was divided into eight lots of 50 ml each and temperature was adjusted to 20±0.1ºC in a water bath. The first lot was kept as control and 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 min 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 if it had deviated.

**3.4.13.2.7.2 Determination of heat stability**

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 for the determination of heat stability of individual milk samples. The tubes were then tilted at the rate of 8 cycles per min 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 min) on Y-axis and pH on X-axis. Heat stability was determined from the maxima of the HCT/pH graph thus obtained.

#### 3.4.13.8 Colour estimation

Hunter colorimeter was used to measure the degree of change in colour produced by addition of vitamin A to fortified milk (containing selected complexes and free vitamin A (oily form)) as compared to control (unfortified) 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.

### 3.4.13 Evaluation of the effect of different processing and storage conditions on stability of vitamin A in milk protein-Vit A complexes and free vitamin A (oily form) fortified milk

#### 3.4.14.1 Estimation of vitamin A content in milk

Estimation of vitamin A content in fortified milk was carried out by the method as described earlier in section 3.4.8.1. However, 1 ml milk sample was used instead of 1ml milk protein-Vit A complex and further steps i.e. saponification, extraction and HPLC conditions remained the same.

#### 3.4.14.2 Effect of heat treatments on stability of added nutrients

Effect of three different heat treatments on the stability of vitamin A in control (unfortified) and vitamin A fortified milk (containing selected complexes and free vitamin A (oily form)) was evaluated. Three different heat treatments were pasteurisation, boiling and sterilization. Stability (%) was analysed by evaluating the vitamin A content before and after the heat treatments. Flow diagrams for preparation of pasteurized, boiled and sterilized milk are illustrated in Figures 3.3, 3.4 and 3.5, respectively.

##### 3.4.14.2.1. Effect of pasteurization on stability of vitamin A

Control (unfortified) and vitamin A fortified milk samples were pasteurized at 63°C/30 min. Samples were cooled and after 2 h of storage, milk samples were analysed for vitamin A content.

##### 3.4.14.2.2 Effect of boiling on stability of vitamin A

Control (unfortified) and vitamin A fortified milk samples were boiled, cooled and finally stored under refrigerated conditions (4-7°C) for 2 h. Samples were further analysed for vitamin A content.

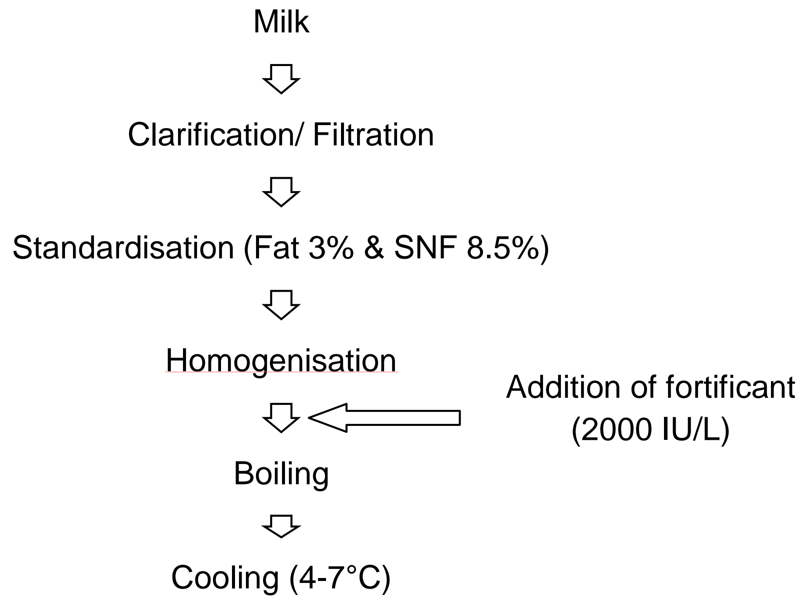



**Figure 3.4: Flow diagram for preparation of vitamin A fortified boiled milk**

### 3.4.14.2.3 Effect of sterilisation on stability of vitamin A

Control (unfortified) and vitamin A fortified milk samples were filled in the transparent glass bottles and sterilized at 121°C for 15 min at 15 psi. Samples were cooled and after 2 h of storage, analysed for vitamin A content.

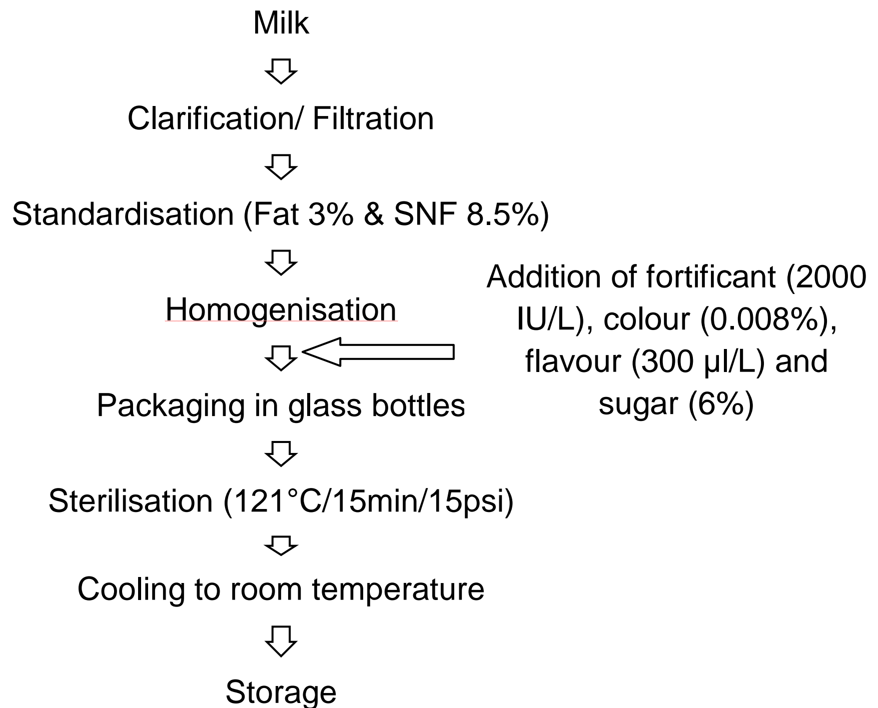



**Figure 3.5: Flow diagram for preparation of vitamin A fortified sterilised milk**

#### 3.4.14.3 Effect of exposure to different light intensities on vitamin A stability

Control (unfortified) and vitamin A fortified pasteurized milk samples were stored in both transparent glass bottles and low density polyethylene (LDPE, thickness 0.07mm) poly pouches. These samples were stored at 5°C in incubator and exposed to different light intensities 1485, 2970 and 4455 lux. Sample was analysed for vitamin A content at 0, 12, 24, 36 and 48 h.

#### 3.4.14.4 Effect of different packaging material on vitamin A stability

Control (unfortified) and vitamin A fortified pasteurized milk samples were stored in transparent glass bottles and LDPE pouches and stored under refrigerated conditions (4-7°C). Samples were analysed for vitamin A content on 0, 3$^{rd}$, 5$^{th}$ and 7$^{th}$ day of storage.

Control (unfortified) and vitamin A fortified sterilized milk samples were stored in transparent glass bottles and stored at room temperature (37°C). Samples were analysed for vitamin A content at an interval of 15 days upto 90 days of storage.

### 3.4.15 *In-vitro* bioavailability of added vitamin A from control (unfortified) and vitamin A fortified milk

Bioavailability of vitamin A from control (unfortified) and vitamin A fortified milk (containing selected complexes and free vitamin A (oily form)) was evaluated using the method of Gupta *et al.* (2015). Bioavailability was estimated according to the method described earlier in section 3.4.11.

### 3.4.16 Statistical analysis

Mean and standard error mean (SEM) were calculated using Microsoft Excel, 2007 (Microsoft Corp., Redmond, WA). Significant difference between values was verified by one way or two way analysis of variance and comparison between means was made by critical difference value (Snedecor & Cochran, 1994).

# CHAPTER –4

# Results and Discussion

# RESULTS AND DISCUSSION

This chapter deals with the various results obtained during the present study. The first phase of study focuses on determining the binding ability and solubility of different milk protein-Vit A complexes prepared from both native and modified (succinylated) milk proteins. Native milk protein-Vit A complexes were prepared using sodium caseinate (NaCas), whey protein concentrate (WPC) and milk protein concentrate (MPC) and the modified milk protein-Vit A complexes were prepared using succinylated sodium caseinate (SNaCas) and succinylated milk protein concentrate (SMPC). Reassembled milk protein-Vit A complexes were prepared using NaCas and SNaCas. The maximum concentration of vitamin A that can be bound to milk protein was selected on the basis of binding ability. Milk protein-Vit A complexes which showed higher binding ability and solubility were chosen for further studies. In the second phase, physicochemical characterization, stability and *in-vitro* bioavailability of selected milk proteins-Vit A complexes was performed. In the third phase, milk was fortified with free vitamin A (oily form) and selected milk protein-Vit A complexes separately. The fortified milk was then evaluated for sensory, physicochemical properties and *in-vitro* bioavailability of added vitamin A. The results of different experiments are listed and discussed under the following heads.

## 4.1 Analysis of milk protein powders:

All milk protein samples (NaCas, MPC and WPC) were analysed for the protein content using Kjeldahl method (AOAC 1970). On the basis of the estimated protein content, these protein powder samples were used for further studies. Table 4.1 shows the protein content in different milk protein samples.

**Table 4.1: Protein content in different milk protein samples**

| Milk protein samples | Protein content (%) |
|---|---|
| Sodium caseinate (NaCas) | 90 |
| Whey protein concentrate (WPC) | 80 |
| Milk protein concentrate (MPC) | 85 |

## 4.2 Preparation of ß-CN

ß-CN was prepared using calcium caseinate and skim milk by following the method of Ward and Bastian (1996) and Huppertz *et al.* (2006), respectively. ß-CN was prepared by three methods (section 3.4.2) as indicated below:

1. Dissolution of calcium caseinate in 10 mM calcium chloride
2. Centrifugation of casein suspension (stored at 5°C for 24 h) obtained from skim milk
3. Addition of calcium chloride to casein suspension (stored at 5°C for 24 h) obtained from skim milk

Yield of ß-CN by different methods is presented in Table 4.2.

**Table 4.2: Yield of ß-CN**

| Different methods | Yield of ß-CN (mg/L of calcium caseinate (3%) or skim milk) |
|---|---|
| Method 1 | 10.2 |
| Method 2 | 11.0 |
| Method 3 | 11.5 |

Purity of obtained ß-CN was evaluated using RP-HPLC by the method of Jahaniaval *et al.* (2000). Chromatogram of ß-CN standard and ß-CN prepared by different methods is depicted in figure 4.1 which showed that ß-CN obtained by these methods had high purity.

Casein is a major milk protein consisting of four fractions i.e. $\alpha_{s1}$-, $\alpha_{s2}$-, β-, and κ-CN in a proportion of 4:1:4:1 (by weight). It can be thought of as amphiphilic block copolymers consisting of blocks with high levels of hydrophobic or hydrophilic amino acid residues. Caseins exhibit a strong tendency to self-assemble into casein micelles because of its amphiphilic property in aqueous solution (Liu and Guo, 2008). ß-CN is the most hydrophobic milk protein. Sullivan *et al.* (1955) reported that the solubility of ß-CN in buffer solution and milk increased as the temperature decreased (<15°C). Rose (1968) also reported that 30% of ß-CN was dissociated from casein micelles at 4°C.

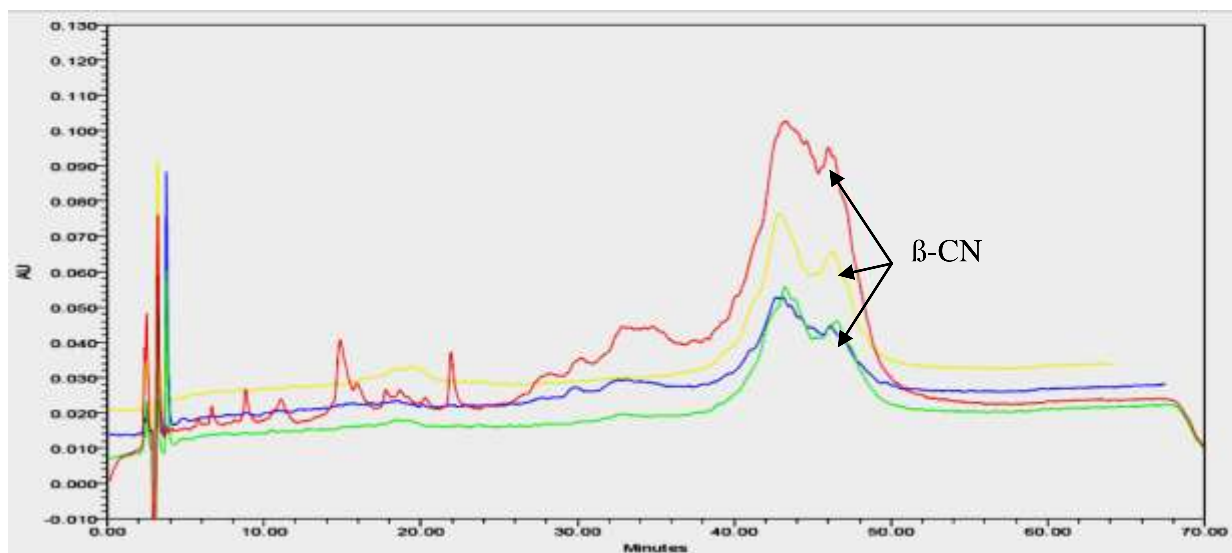



**Fig 4.1: ß-CN chromatogram (a) to (d) from upper to lower (a) ß-CN standard, (b) ß-CN prepared by method 3, (c) ß- CN prepared by method 1 and (d) ß-CN prepared by method 2**

On the basis of dissociation of ß-CN at low temperature, several methods have been reported i.e. by filtration of sodium caseinate through filter paper (Famelart *et al.* 1989), microfiltration (Famelart and Surel, 1994), ultrafiltration (Murphy and Fox, 1991) or from cold-renneted calcium caseinate (Allen *et al.* 1985). ß-CN solubilises at low temperature of storage which is due to the weakening of hydrophobic bonds (Law, 1996; Pierre and Brule, 1981) and resulted in high solubility of ß-CN (Payens and Van Marwijk, 1963; Sullivan *et al.* 1955). Presence of calcium changes the solubility of ß-CN. The isolated ß-CN is sensitive to calcium and precipitates in the presence of 10 mM calcium chloride at 37°C (Dickson and Perkins, 1971; Imafidon and Ng-Kwai-Hang, 1992). The amount of ß-CN that precipitates depends on pH, temperature and calcium concentration (Imade *et al.* 1977; Imafidon and Ng-Kwai-Hang, 1992; Parker and Dalgleish, 1981).

Most commonly used method for separation of four major caseins is anion exchange chromatography, which uses high concentration of chaotropic agents i.e. urea (>3.3 M). This technique can be easily scaled up but after the exhaustic dialysis also toxic material will be present with fractions, hence, not suitable for use in foods. Therefore, this method was not used for the preparation of ß-CN (Law and Leaver, 2002).

However, the yield of the ß-CN was very low (10.2-11.5 mg/L of skim milk and calcium caseinate), hence, ß-CN was not considered for the further analysis as it will not be commercially viable to manufacture complexes involving ß-CN.

### 4.3 Succinylation of milk protein

A detailed study on succinylation was carried out to elaborate the reaction between succinic anhydride and proteins. Optimization of the concentration of succinic anhydride was carried out to obtain a maximum degree of succinylation of different proteins. Amino acids like Tyr, His, Ser, Thr and Cys can also undergo succinylation upon reaction with succinic anhydride but, the reaction rate is slower and the product formed is less stable than that with Lys (Thompson and Reyes, 1980). Hence, the content of lysine was considered to express the degree of succinylation. Shilpashree *et al.* (2015a, b) reported that highest degree of succinylation of sodium caseinate and milk protein concentrate was achieved at a concentration of 4 moles of succinic anhydride/mole of lysine. Hence, concentration of succinic anhydride was varied from 2.0 to 4.0 moles of succinic anhydride/mole of lysine. The content of lysine was fixed at 50 mM of lysine content of different milk proteins and succinic anhydride concentration was varied from 2.0 to 4.0 moles of succinic anhydride/mole of lysine.

#### 4.3.1 Preparation of SNaCas

Impact of different levels of succinic anhydride on lysine content of protein and degree of succinylation is depicted in table 4.3. It can be inferred from the figure 4.2 that the degree of succinylation was highest when the concentration of succinic anhydride reaches 3.2 moles of succinic anhydride/mole of lysine. Even though, there was an increase in degree of succinylation beyond 3.2 moles of succinic anhydride/mole of lysine, the difference was non significant ($p>0.05$) indicating that saturation point of succinylation of casein in NaCas occurred at 3.2 moles of succinic anhydride/mole of lysine. Increase of succinic acid concentration from 3.2 to 4.0 moles of succinic anhydride /mole of lysine resulted in only 0.51% increase in degree of succinylation. Thus, 3.2 moles of succinic anhydride/mole of lysine was optimized for preparation of SNaCas.

**Table 4.3: Succinylation of NaCas and its effect on degree of succinylation**

| Mole of succinic anhydride/mole of lysine | Lysine content after succinylation (g/100 g protein) | Degree of succinylation | Difference between degree of succinylation |
|---|---|---|---|
| 2.0 | 2.64±0.13 | 62.58±1.25 | - |
| 2.2 | 2.31±0.20 | 69.39±1.16 | 6.81±0.18[a] |
| 2.4 | 2.09±0.11 | 76.32±2.10 | 6.93±0.11[b] |
| 2.6 | 1.82±0.13 | 81.86±1.62 | 5.54±1.63[c] |
| 2.8 | 1.96±0.13 | 87.02±2.22 | 5.16±1.23[d] |
| 3.0 | 1.34±0.22 | 91.20±1.23 | 4.18±1.15[e] |
| 3.2 | 1.27±0.14 | 94.91±2.2* | 3.71±1.28[f] |
| 3.4 | 1.06±0.12 | 95.26±18 | 0.35±0.10[g] |
| 3.6 | 0.72±0.24 | 95.33±24 | 0.07±0.18[g] |
| 3.8 | 0.60±0.17 | 95.38±1.25 | 0.05±0.15[g] |
| 4.0 | 0.49±0.12 | 95.42±1.18 | 0.04±0.11[g] |

Data are presented as means ± SEM (n=3).
[a-b]Means within columns with different superscript are significantly different (p<0.05) from each other.
*Highest degree of succinylation of caseins was achieved at this concentration of succinic anhydride (3.2 moles of succinic anhydride/mole of lysine)

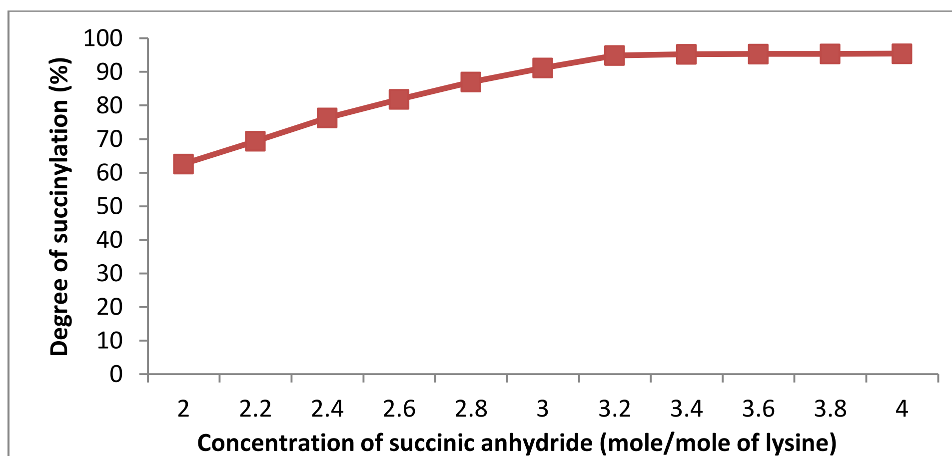



**Fig 4.2: Degree of succinylation of NaCas as affected by concentration of succinic anhydride (mole/mole of lysine)**

### 4.3.2 Preparation of SMPC

Impact of different levels of succinic anhydride on lysine content of protein and degree of succinylation is depicted in table 4.4. It can be inferred from the figure 4.3 that the degree of succinylation was highest when the concentration of succinic anhydride reaches 3.2 moles of succinic anhydride/mole of lysine. Even though, there was an increase in degree of succinylation beyond 3.2 moles of succinic anhydride/mole of

lysine, the difference was non significant (p>0.05) indicating that saturation point of succinylation of milk protein in MPC occurred at 3.2 moles of succinic anhydride/mole of lysine. Increase of succinic acid concentration from 3.2 to 4.0 moles of succinic anhydride /mole of lysine resulted in only 0.39% increase in degree of succinylation. Thus, 3.2 moles of succinic anhydride/mole of lysine was optimized for preparation of SNaCas.

**Table 4.4: Succinylation of MPC and its effect on degree of succinylation**

| Mole of succinic anhydride/mole of lysine | Lysine content after succinylation (g/100 g protein) | Degree of succinylation | Difference between degree of succinylation |
|---|---|---|---|
| 2.0 | 2.04±0.10 | 74.78±1.36 | - |
| 2.2 | 1.97±0.20 | 77.92±1.12 | 3.14±0.14$^{a}$ |
| 2.4 | 1.54±0.18 | 81.41±2.20 | 3.49±0.38$^{b}$ |
| 2.6 | 1.38±0.12 | 85.05±1.16 | 3.64±0.11$^{c}$ |
| 2.8 | 1.19±0.19 | 88.31±2.17 | 3.26±0.18$^{d}$ |
| 3.0 | 1.05±0.11 | 91.42±1.22 | 3.11±0.22$^{e}$ |
| 3.2 | 0.90±0.23 | 94.72±1.13* | 3.30±0.29$^{f}$ |
| 3.4 | 0.82±0.14 | 94.75±2.24 | 0.03±0.42$^{g}$ |
| 3.6 | 0.69±0.12 | 94.90±1.12 | 0.15±0.12$^{g}$ |
| 3.8 | 0.61±0.19 | 94.98±1.27 | 0.08±0.14$^{g}$ |
| 4.0 | 0.55±0.15 | 95.11±1.22 | 0.13±0.06$^{g}$ |

Data are presented as means ± SEM (n=3).
$^{a-b}$Means within columns with different superscript are significantly different (p<0.05) from each other.
*Highest degree of succinylation of milk protein was achieved at this concentration of succinic anhydride (3.2 moles of succinic anhydride/mole of lysine)

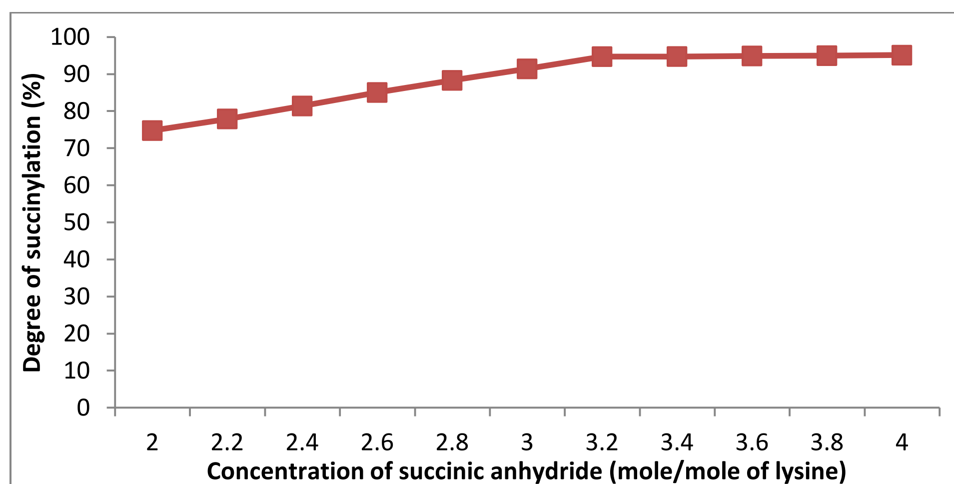



**Fig 4.3: Degree of succinylation of MPC as affected by concentration of succinic anhydride (mole/mole of lysine)**

### 4.4 Preparation of milk protein-Vit A complexes:

Milk protein-Vit A complexes were prepared using different native (NaCas, MPC and WPC) and modified (SNaCas and SMPC) milk proteins. Reassembled milk protein-Vit A complexes were prepared using NaCas and SNaCas. The amount of different milk proteins required for the preparation of 5% protein solution is presented in table 4.5, these proteins were dissolved in 50 ml of double distilled water for preparation of complexes utilizing stirring (ST) and sonication (SO).

**Table 4.5: Amount of protein required for preparation of milk protein-Vit A complexes**

| Protein powder sample | Protein (%) | Quantity of protein powder required for 5% protein solution |
|---|---|---|
| Sodium caseinate (NaCas) | 90 | 5.55 gm |
| Milk protein concentrate (MPC) | 85 | 5.88 gm |
| Whey protein concentrate (WPC) | 80 | 6.25 gm |
| Succinylated sodium caseinate (SNaCas) | 85 | 5.88 gm |
| Succinylated milk protein concentrate (SMPC) | 81 | 6.17 gm |

Preparation of reassembled sodium caseinate-Vit A (RNaCas-VA) and reassembled succinylated sodium caseinate-Vit A (RSNaCas-VA) complexes were done using NaCas and SNaCas, respectively. The yield of milk protein-Vit A complexes was expressed on the basis of gm of protein powder used for the preparation of complexes (Table 4.6).

The yield (%) of milk protein-Vit A complexes were not affected by method of preparation of these complexes. Difference in the yield of complexes mainly depends upon the moisture content of the samples after lyophilisation. Reassembled complexes showed higher yield due to the incorporation of $K_2HPO_4$ and $CaCl_2$ in the complexes.

### 4.5 Standardization of analytical conditions for extraction and estimation of total vitamin A

Milk protein-Vit A complexes were prepared and vitamin A content in the samples were analysed using the method of Kaushik *et al.* (2014a). However, the estimation of

**Table 4.6: Yield of milk protein vitamin A complexes**

| Different complexes | Yield (%) (on weight basis) |
|---|---|
| **By stirring (ST)** | |
| Sodium caseinate-Vit A (NaCas-VA ST) | 94.48 |
| Milk protein concentrate-Vit A (MPC-VA ST) | 91.61 |
| Whey protein concentrate-Vit A (WPC-VA ST) | 91.03 |
| Succinylated sodium caseinate-Vit A (SNaCas-VA ST) | 91.56 |
| Succinylated milk protein concentrate-Vit A (SMPC-VA ST) | 90.66 |
| **By sonication (SO)** | |
| Sodium caseinate-Vit A (NaCas-VA SO) | 92.90 |
| Milk protein concentrate-Vit A (MPC-VA SO) | 90.81 |
| Whey protein concentrate-Vit A (WPC-VA SO) | 90.24 |
| Succinylated sodium caseinate-Vit A (SNaCas-VA SO) | 90.81 |
| Succinylated milk protein concentrate-Vit A (SMPC-VA SO) | 87.21 |
| **Reassembling method** | |
| Reassembled sodium caseinate-Vit A (RNaCas-VA) | 113.08 |
| Reassembled succinylated sodium caseinate-Vit A (RSNaCas-VA) | 113.78 |

vitamin A involved standardization of saponification method, time required for saponification, extraction solvent, HPLC mobile phases and injection volume.

#### 4.5.1 Saponification

Saponification was carried out according to the method of Kaushik *et al.* (2014a) with slight modification. Samples were prepared by two methods:

a. In the first method, sample was treated with 2.5% $NH_4OH$ and 0.1 N HCl and
b. In the second method, sample was not treated with both the reagents

Samples treated with 2.5% $NH_4OH$ and 0.1 N HCl showed higher area of HPLC peak (ultimately greater recovery) as compared to samples which were not treated with these reagents. Hence, saponification process involving $NH_4OH$ and HCl was selected for further evaluation of the samples. $NH_4OH$ and HCl were used to break the bonds formed between the milk protein and vitamin A in the complex. Figure 4.4 and table 4.7

depicted the effect of $NH_4OH$ and HCl treatment on the recovery of vitamin A. Variation in time of saponification (30 min, 45 min and 60 min) was also assayed for its effect on recovery of vitamin A. Maximum recovery of vitamin A was observed after 30 min saponification (Fig 4.5 and Table 4.8), hence, 30 min saponification was selected for further analysis. Alcoholic pyrogallol was used as an antioxidant and potassium hydroxide was used for the saponification of the milk protein-Vit A complexes which convert vitamin A ester to vitamin A (Retinol). Sequence of $NH_4OH$ and HCl addition did not affect the extraction of vitamin A from the samples. However, in this process the sequence of $NH_4OH$ addition was followed by HCl addition.

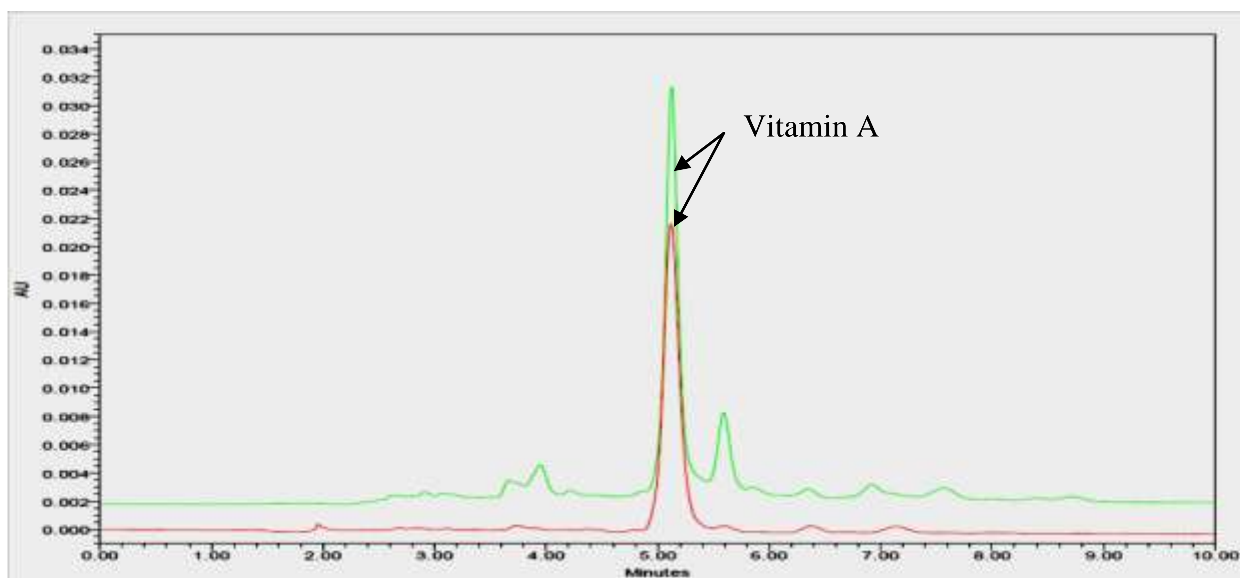



**Fig 4.4: Effect of $NH_4OH$ and HCl treatment on recovery of vitamin A, (a) to (b) from upper to lower, (a) Sample treated with $NH_4OH$ and HCl (b) Sample not treated with $NH_4OH$ and HCl**

Vitamin A analysis was carried out using different analytical techniques (colorimetric, spectrophotometric and HPLC) and also with different extraction procedures (AOAC, 2005; DeVries and Silvera, 2002; Hite, 2003; Sullivan, 2012; McMahon *et al.* 2013).

**Table 4.7: Effect of $NH_4OH$ and HCl treatment on recovery of vitamin A**

| Treatment | Area (µV*sec) |
|---|---|
| Sample treated with $NH_4OH$ and HCl | 228610 |
| Sample not treated with $NH_4OH$ and HCl | 222588 |

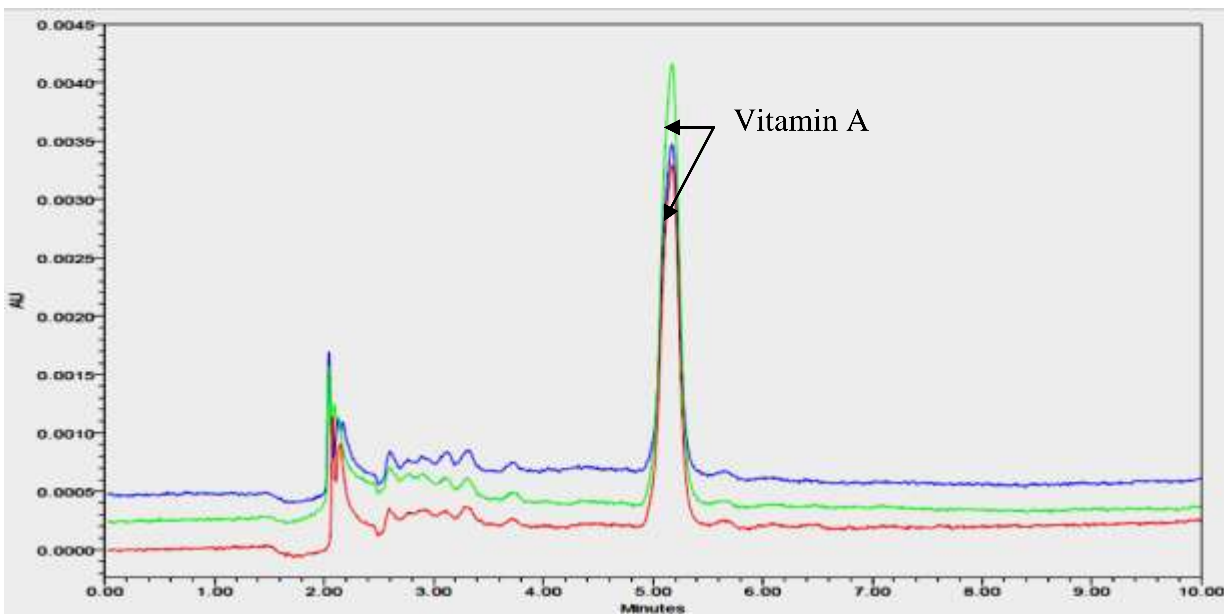



**Fig 4.5: Effect of saponification time on recovery of vitamin A, (a) to (c) from upper to lower, (a) 60 min, (b) 30 min and (c) 45 min**

Different workers Butt *et al.* (2007), Chavez-Servin *et al.* (2006), Faulkner *et al.* (2000), Hite (2003), Johnson-Davis *et al.* (2009), Kim *et al.* (2000), Lee *et al.* (2000), Thompson *et al.* (1980), Wegmuller *et al.* (2006), Wang and Huang (2002) directly measured the vitamin A as retinol, retinyl palmitate, retinyl acetate and different isomers of these in fortified cookies, cosmetic products, pharmaceuticals, corn flakes, fluid milk, margarine, mixed feeds, premixes, human and pet foods, fortified rice, Infant and pediatric formula, blood serum and different foods without saponification and direct extraction of vitamin A in hexane and ethanol solvents. Antioxidants were not used during the extraction of vitamin A. Lee *et al.* (2000) also reported saponification as severe extraction process which may result in degradation and isomerisation of the native compound.

**Table 4.8: Effect of saponification time on recovery of vitamin A**

| Saponification time (min) | Area (µV*sec) |
|---|---|
| 30 | 42627 |
| 45 | 34773 |
| 60 | 31014 |

Kaushik *et al.* (2014a) reported that encapsulated vitamin $D_2$ (cold water soluble) require additional treatment of $NH_3$ and HCl for disruption of coating material and assistance in proper extraction of vitamin $D_2$ from microcapsules. In the present study, free vitamin A (oily form) was bound to milk protein, hence, additional steps of $NH_3$ and HCl treatment were required for proper extraction of vitamin A from the complexes and free vitamin A (oily form).

Saponification converts ester form of vitamin A (retinyl palmitate, retinyl acetate) to alcohol (retinol) form, hence, retinol was measured in all the samples rather than vitamin A palmitate and acetate (Thompson *et al.* 1949). Kuhn *et al.* (2008), Maguer and Jackson (1983), Medrano *et al.* (1994), Pallavi *et al.* (2014), Thompson *et al.* (1972), Zahar and Smith (1990) estimated vitamin A in polymeric diets, UHT processed milk, milk, peanut *chikki* and dairy products after saponification with KOH (aqueous or alcoholic) and with the use of different antioxidants (i.e. hydroquinone, ascorbic acid, butylated hydroxyl toluene and alcoholic pyrogallol).

### 4.5.2 Extraction

Vitamin A was extracted from saponified samples using different solvents (section 3.4.5.1.2) i.e. (a) Chloroform and methanol, (b) hexane and alcohol, (c) hexane, acetone and alcohol (2:1:1) and (d) hexane, alcohol and saturated NaCl. Among all the extraction solvents used, extraction with 4 ml of chloroform:methanol in the ratio of 1:2 and further addition of 1 ml chloroform gave maximum extraction of the vitamin A (Fig 4.6 and table 4.9). This extraction method was selected for further analysis of the samples. Various researchers reported the use of hexane, petroleum ether and diethyl ether for the extraction of vitamin A (Chavez-Servin *et al.* 2006; Kuhn *et al.* 2008; Maguer and Jackson, 1983; Medrano *et al.* 1994; Pallavi *et al.* 2014; Thompson *et al.* 1972; Zahar and Smith, 1990). Kazmi *et al.* (2007) used chloroform:methanol mixture for the extraction of vitamin $D_3$ from processed dairy products.

### 4.5.3 HPLC conditions

Vitamin A gave maximum absorption at 325 nm, hence, this wavelength was selected for the analysis of vitamin A by RP-HPLC. Various researchers have reported

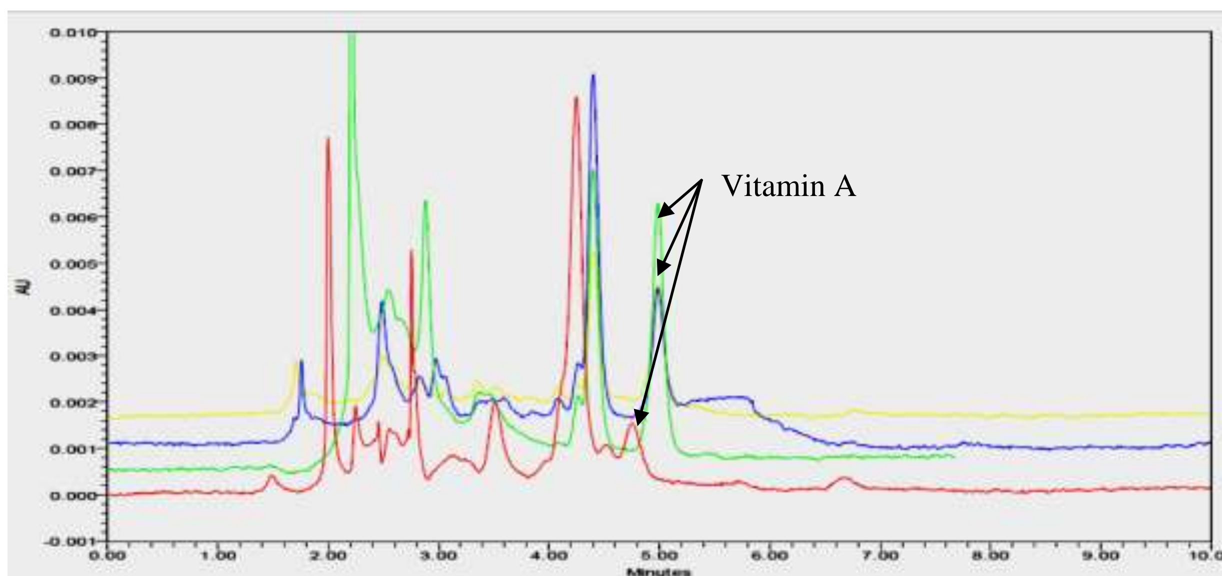



**Fig 4.6: Effect of different extraction solvents on recovery of vitamin A, (a) to (d) from upper to lower, (a) Hexane and alcohol, (b) Hexane, acetone and alcohol, (c) Chloroform and methanol and (d) Hexane, alcohol and saturated NaCl**

**Table 4.9: Effect of different extraction solvents on recovery of vitamin A**

| Extraction Solvent | Area (µV*sec) |
|---|---|
| Hexane and alcohol | 21226 |
| Hexane, acetone and alcohol | 20718 |
| Chloroform and methanol | 41639 |
| Hexane, alcohol and saturated NaCl | 7737 |

the use of various wavelength i.e. 313 nm (DeVries and Silvera, 2002; Zahar and Smith, 1990), 320 nm (Pallavi *et al.,* 2014), 325 nm (Hite, 2003; Johnson-Davis *et al.,* 2009; Sullivan, 2012; Mohan *et al.,* 2013; McMahon *et al.,* 2013; Zahar and Smith 1990), 327 nm (Kim *et al.,* 2000) and 328 nm (DeVries and Silvera, 2002; Faulkner *et al.,* 2000) for estimation of vitamin A. However, the most widely used wavelength for the estimation of vitamin A is 325 nm. Two different mobile phases, acetonitrile: methanol: chloroform (88:8:4) and acetonitrile: methanol: water (49.5:49.5:1) were also selected for analysis of vitamin A. Among these two, acetonitrile: methanol: water (49.5:49.5:1) gave better resolution as compared to acetonitrile: methanol: chloroform (88:8:4). Figure 4.7 and

table 4.10 depicted the effect of different mobile phases on elution of vitamin A. Pallavi *et al.* (2014) used acetonitrile, methanol and chloroform in the ratio of 65:25:10 as

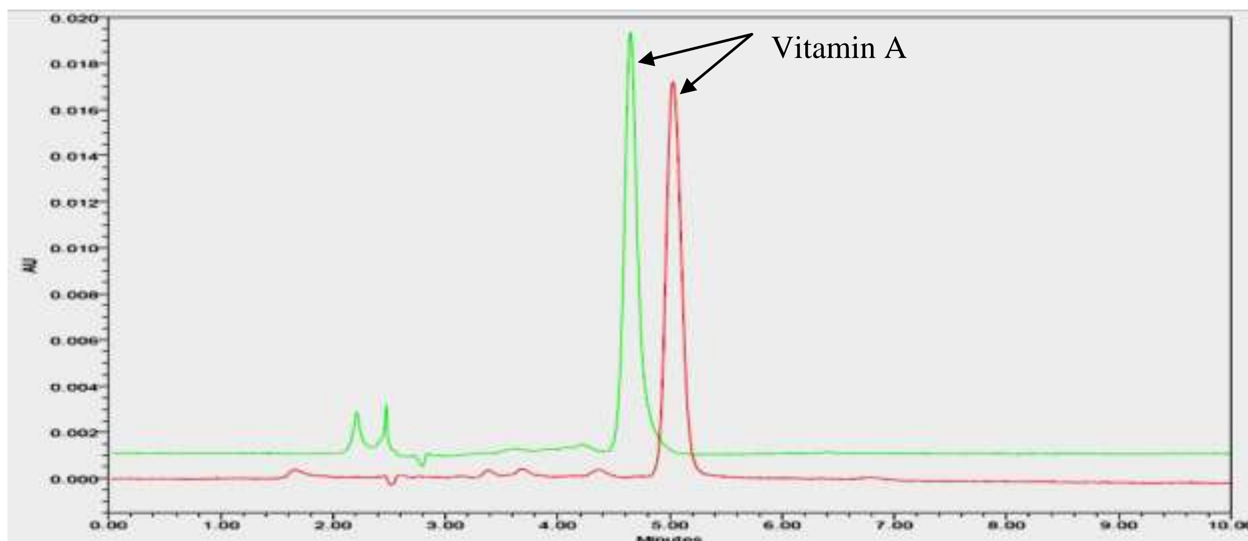



**Fig 4.7: Effect of different mobile phases, (a) to (b) from upper to lower, (a) Acetonitrile: Methanol: Chloroform (88:8:4) and (b) Acetonitrile: Methanol: Water (49.5:49.5:1)**

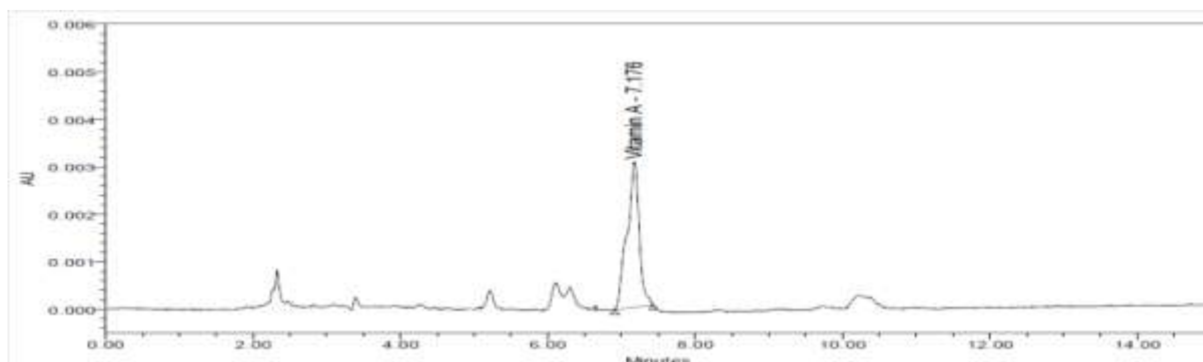
mobile phase for vitamin A estimation in fortified chikki. Medrano *et al.* (1994), Zahar and Smith (1990) reported the use of methanol:water in the ratio of 98:2 and 95:5 as mobile phase for estimation of vitamin A.

**Table 4.10: Effect of different mobile phases**

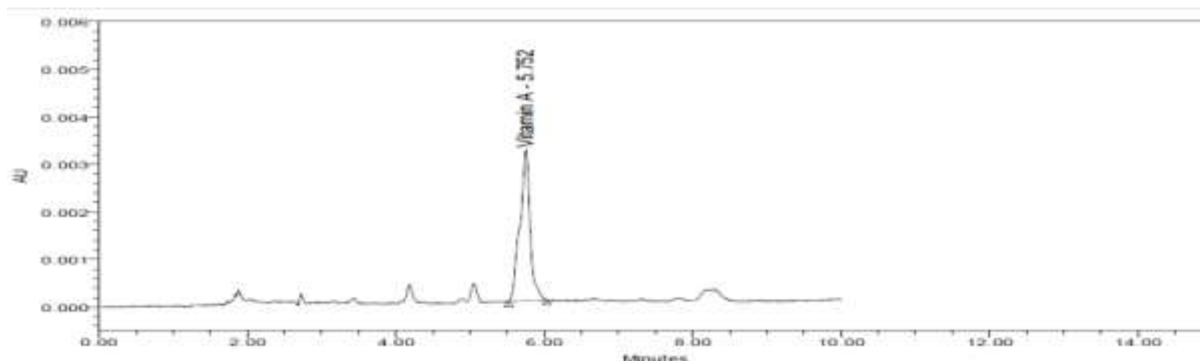
| Mobile phase | Area (μV*sec) |
|---|---|
| Acetonitrile: Methanol: Chloroform (88:8:4) | 155001 |
| Acetonitrile: Methanol: Water (49.5:49.5:1) | 162510 |

Effect of different flow rates on the elution of vitamin A was monitored and different retention times were observed due to variation in flow rates. Increase in flow rate resulted in decrease in retention time. The retention time was found to be 7.176, 5.752 and 4.809 min under the flow rate of 0.8, 1 and 1.2 ml/min, respectively (Fig 4.8). The flow rate of 1ml/min resulted in high resolution of peak, hence, this flow was selected for further analysis. Pallavi *et al.* (2014) and Medrano *et al.* (1994) used a flow rate of 1 ml/min, however, Zahar and Smith (1990) used a flow rate of 0.8 ml/min for quantification of vitamin A through HPLC. Effect of different injection volumes (20 μl,

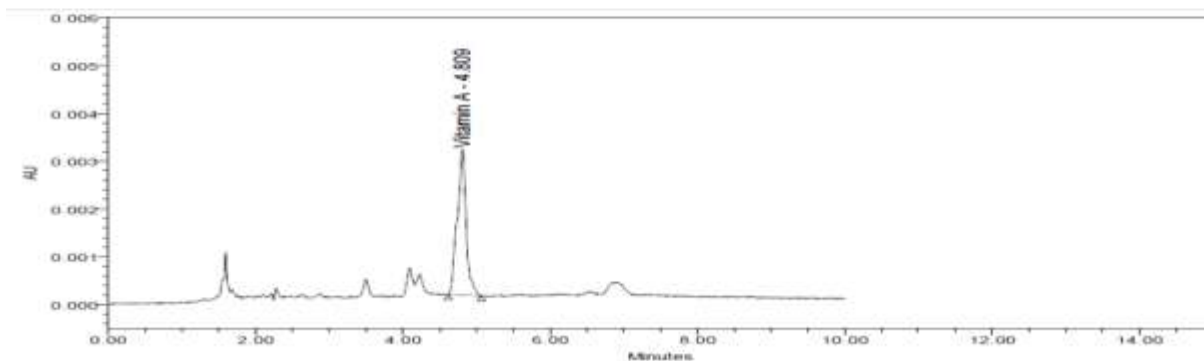
100 µl and 200 µl) on the analysis of vitamin A was also evaluated and found that 20 µl injection volume resulted in measurable area with better peak definition, hence this injection volume was selected for further analysis.




(a)




(b)




(c)

**Fig 4.8: Effect of different flow rate on the retention time of vitamin A (a) 0.8 ml/min, (b) 1 ml/min and (c) 1.2 ml/min**

### 4.5.4 Reconstitution solvent

Mobile phase and methanol were used as reconstitution solvent for the estimation of vitamin A. Mobile phase (acetonitrile:methanol:water 49.5:49.5:1) and

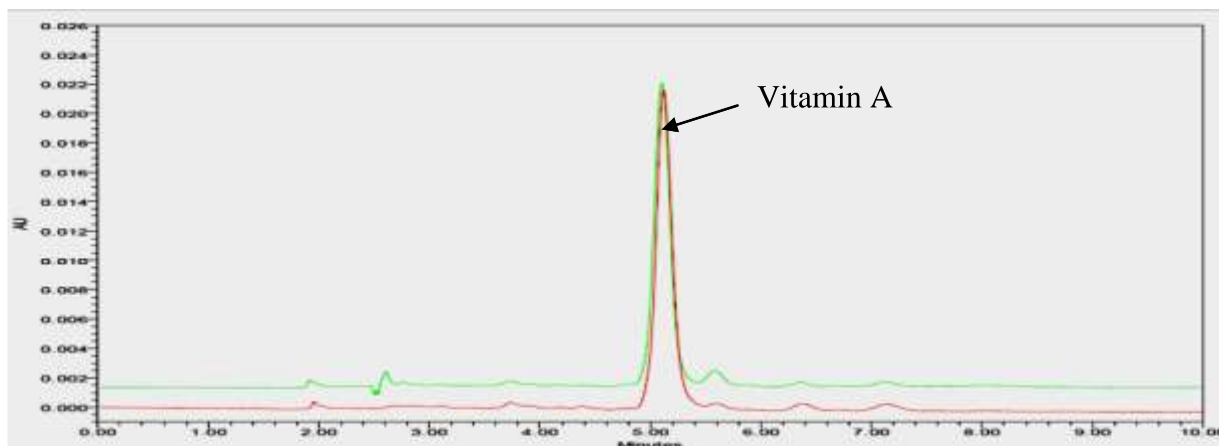



**Fig 4.9: Effect of different reconstitution solvents, (a) to (b) from upper to lower, (a) methanol and (b) mobile phase (Acetonitrile: Methanol: Water 49.5:49.5:1)**

methanol gave similar resolution to the peak of vitamin A, hence, mobile phase was selected as reconstitution solvent for the estimation of vitamin A (Fig 4.9 and Table 4.11). Different researchers reported different reconstitution solvents i.e. petroleum ether (Thompson *et al.* 1972), hexane (Faulkner *et al.,* 2000; Kim *et al.,* 2000; Lee *et al.,* 2000; Maguer and Jackson, 1983; Pallavi *et al.* 2014; Wegmuller *et al.,* 2006;), alcohol (Johnson-Davis *et al.,* 2009) and methanol (Zahar and Smith, 1990; Medrano *et al.,* 1994) for analysis of vitamin A. Kaushik *et al.* (2014a) reported that methanol as reconstitution solvent gave higher area as compared to mobile phase for the estimation of vitamin $D_2$. Kazmi *et al.* (2007) reported the use of mobile phase and Perales *et al.* (2005) reported the use of methanol as reconstitution solvent for the estimation of vitamin $D_3$.

**Table 4.11: Effect of different reconstitution solvents**

| Reconstitution solvents | Area (µV*sec) |
|---|---|
| Methanol | 212185 |
| Mobile phase | 223345 |

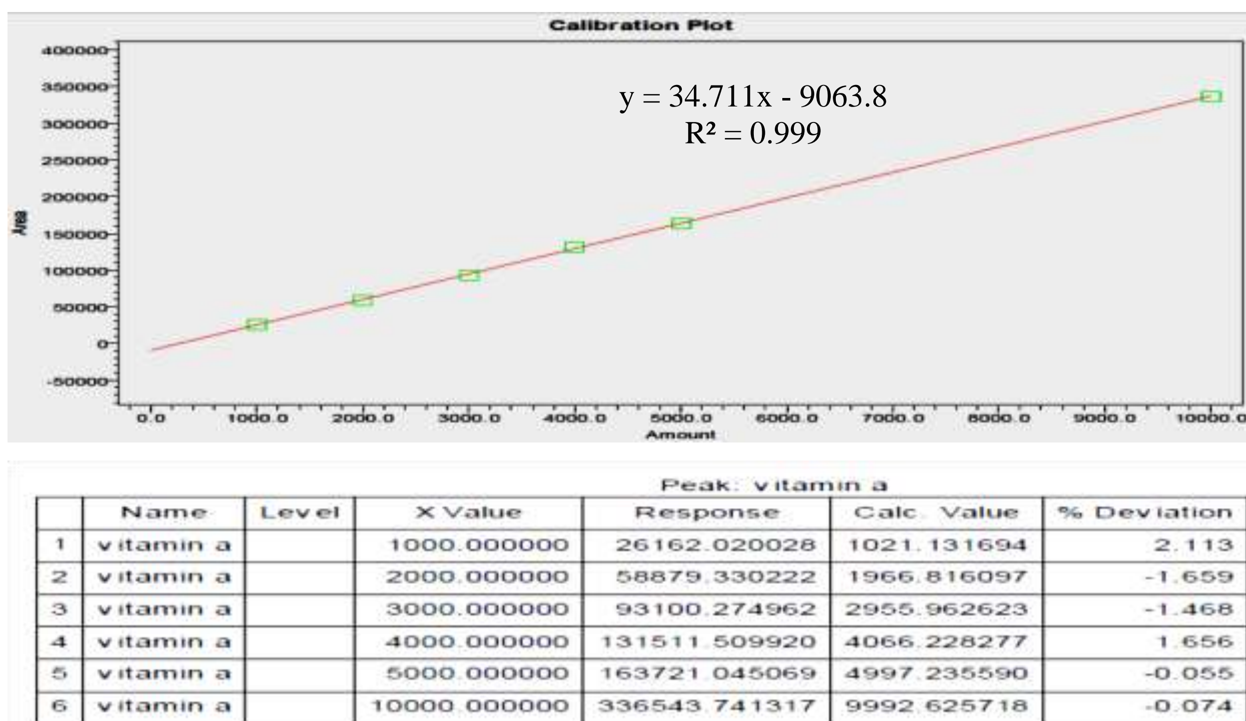
### 4.5.5. Standard curve

Standard curve was prepared using the optimized HPLC conditions. Chromatographic conditions i.e. mobile phase (acetonitrile: methanol: water in the ratio 49.5:49.5:1), injection value (20 µl), flow rate (1 ml/min) and wavelength (325 nm) were used for preparation of standard curve. 13.2 mg retinol as vitamin A standard 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 4356000 IU/L. Six point calibration curve was prepared using standard solutions of concentration 1000, 2000, 3000, 4000, 5000 and 10000 IU/L. Figure 4.10 showed the calibration curve of vitamin A. 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 4.11 depicts the chromatograms of standard vitamin A.

## 4.6 Standardization of analytical conditions for extraction and estimation of unbound vitamin A

Few studies are available in literature regarding the quantitative estimation of ability of protein to bind with different compounds. Various researchers reported the binding of vitamin and other hydrophobic compounds to protein on the basis of fluorescence spectroscopic studies (Esmaili *et al.* 2011; Li *et al.* 2013; Liang *et al.* 2011; Perez *et al.* 2014a; Yazdi and Corredig 2012; Yuksel *et al.* 2010). Various methods were tried for estimation of unbound vitamin A in milk protein-Vit A complexes in the present work.




Peak: vitamin a

| | Name | Level | X Value | Response | Calc. Value | % Deviation |
|---|---|---|---|---|---|---|
| 1 | vitamin a | | 1000.000000 | 26162.020028 | 1021.131694 | 2.113 |
| 2 | vitamin a | | 2000.000000 | 58879.330222 | 1966.816097 | -1.659 |
| 3 | vitamin a | | 3000.000000 | 93100.274962 | 2955.962623 | -1.468 |
| 4 | vitamin a | | 4000.000000 | 131511.509920 | 4066.228277 | 1.656 |
| 5 | vitamin a | | 5000.000000 | 163721.045069 | 4997.235590 | -0.055 |
| 6 | vitamin a | | 10000.000000 | 336543.741317 | 9992.625718 | -0.074 |

**Fig 4.10: Calibration curve of Vitamin A (Retinol)**

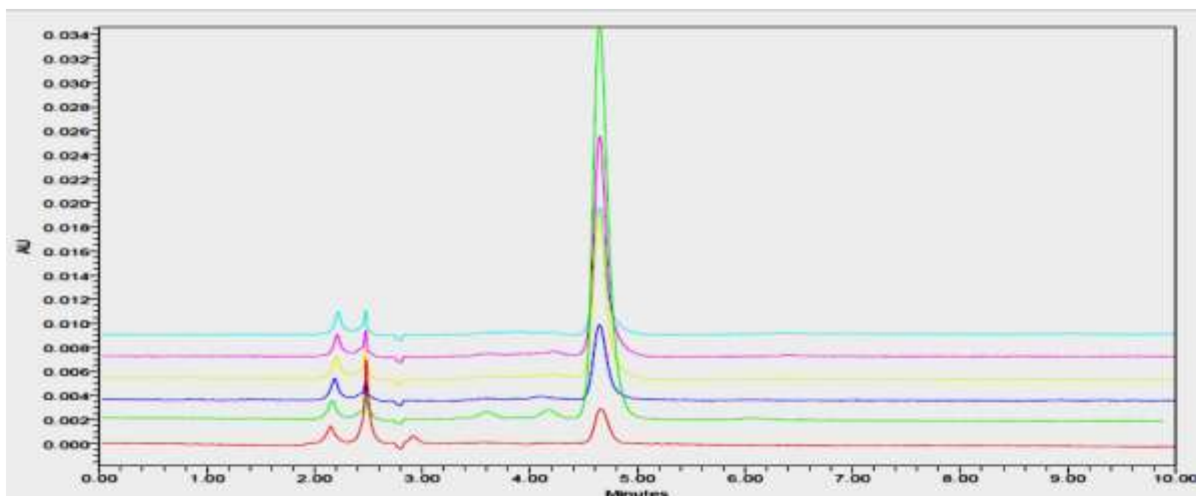



**Fig 4.11: Liquid chromatogram of standard vitamin A, (a) to (f) from upper to lower, (a) 3000 IU/L, (b) 5000 IU/L, (c) 4000 IU/L, (d) 2000 IU/L, (e) 10000 IU/L and (e) 1000 IU/L**

### 4.6.1 Centrifugation method

It was assumed that high speed centrifugation will result in the precipitation of milk protein and bound vitamin will also precipitate together with milk protein. Samples were centrifuged at 25000 g for 30 min at 20°C, however, complete precipitation of milk protein was not observed. Supernatant was hazy which indicated the presence of proteins in the supernatant, hence this method was not used for estimation of unbound vitamin A in the complexes.

Several researchers reported the use of centrifugation for the estimation of ability of protein to bind with different compounds. α-tocopherol was encapsulated with ß-lactoglobulin and encapsulation efficiency was measured by quantifying the free α-tocopherol after centrifugation at 12000 rpm for 30 min (Somchue *et al.,* 2009). Chevalier-Lucia *et al.* (2011) studied the binding of phosphocasein dispersion to α-tocopherol acetate (α-TA) and used high speed centrifugation at 23160 g for 30 min at 20°C for separation of bound α-TA from free α-TA. Phosphocasein dispersion was bound to curcumin by dynamic high pressure processing and high speed centrifugation was used at 16000 g for 5 min for separation of bound curcumin from free curcumin (Benzaria *et al.* 2013). Menendez-Aguirre *et al.* (2014) used high speed centrifugation (25000 g for 1 h at 20°C) for separation of small soluble micelles from large micelles

and measured vitamin $D_2$ in both the phases. Ionically cross linked milk protein nanoparticles were prepared as flutamide carrier and incorporation efficiency of these nanoparticles were measured by estimating the free flutamide after centrifugation at 20000 g for 30 min at 10°C (Elzoghby *et al*. 2013). Haratifar and Corredig (2014) used high speed centrifugation (14000 g for 5 min) after precipitation of casein micelles-tea catechin complex with acetic acid.

### 4.6.2 UF method

UF was carried out according to the method described by Shilpashree *et al.* (2016) with slight modification in the speed of centrifugation. Control sample was prepared by dissolving vitamin A in double distilled water. Control and milk protein-Vit A complexes samples were centrifuged at 18000 g rather than 12000 g to avoid clogging of UF membrane. Supernatant obtained after centrifugation was subjected to UF and vitamin A content was estimated in the permeate. Vitamin A was not recovered in the permeate after UF of different samples (including control), hence, UF method was not used for further analysis. Semo *et al.* (2007) used UF method for separation of free vitamin $D_2$ from casein bound vitamin $D_2$ and could not recover vitamin $D_2$ in permeate.

### 4.6.3 Isoelectric precipitation

Isoelectric precipitation results in changes in the protein structure, hence, may affect binding of vitamin to the milk protein. Due to the high impact of isoelectric precipitation on milk protein structure (Phadungath, 2005), this method was not used for analyzing the unbound vitamin A.

Zimet *et al.* (2011) used isoelectric precipitation method (0.1 N HCl) for precipitation of reassembled casein micelles and casein nanoparticles which was bound to ω-3 polyunsaturated (DHA) fatty acid. Diarrassouba *et al.* (2015) also used isoelectric precipitation method for analysis of vitamin $D_3$ binding ability of ß-lactoglobulin, however, they used glucono-δ-lactone for adjustment of pH.

### 4.6.4 Ammonium sulphate precipitation

Milk protein-Vit A complexes were precipitated with ammonium sulphate and vitamin A content was analysed in both pellet and supernatant. Supernatant contained

high concentration of ammonium sulphate and during saponification potassium hydroxide might react with ammonium sulphate and precipitate as ammonium hydroxide and potassium sulphate, hence, potassium hydroxide was not available for saponification**.** Therefore, estimation of vitamin A in supernatant needed more quantity of potassium hydroxide (60%) for saponification. Different quantities i.e. 1 ml, 2 ml, 3 ml, 4 ml, 5 ml, 10 ml, 15ml and 20 ml of KOH (60%) were used for estimation of vitamin A content in the supernatant. 5 ml KOH (60%) gave the optimum results, however, repeatability of the results was not observed. Hence, vitamin A content was estimated in pellet and total sample. Pellet obtained in the above precipitation was redispersed in 10 ml double distilled water and 1 ml of this sample was used for the analysis of bound vitamin A. The sample was further analysed as described in section 3.4.8.1.

Blayo *et al.* (2014) used whey protein and phosphocasein micelles for binding of retinyl acetate and binding ability was evaluated by estimating the free retinyl acetate after precipitation of protein with ammonium sulphate.

## 4.7 Selection of milk protein-Vit A complexes

### 4.7.1 Recovery of vitamin A from milk protein-Vit A complexes

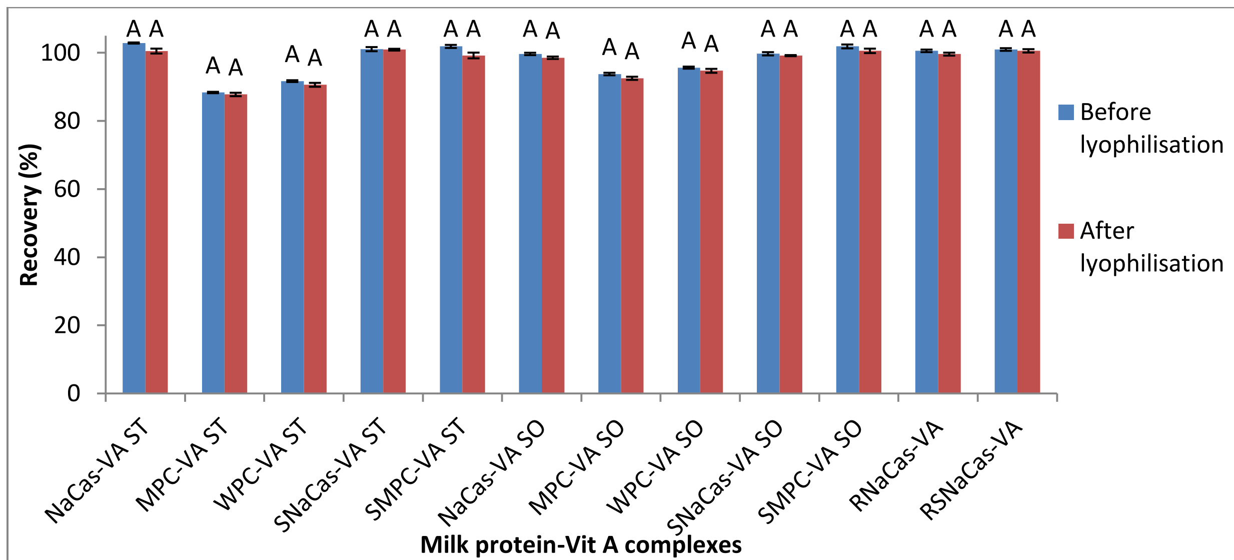
Different milk protein-Vit A complexes were prepared as described earlier in section 3.4.4. Recovery of vitamin A was estimated before and after lyophilisation of the milk protein-Vit A complexes. All the milk protein-Vit A complexes showed non significant difference ($p>0.05$) in recovery of vitamin A before and after lyophilisation of the samples (Table 4.12and Fig 4.12).

Lower recovery was observed for MPC-VA and WPC-VA complexes, however, higher recovery was observed for NaCas-VA, SNaCas-VA, SMPC-VA, RNaCas-VA and RSNaCas-VA complexes. Highest recovery was observed for RSNaCas-VA complex both before and after lyophilisation of the samples (Fig 4.13 (a) and (b)). Difference in the recovery of vitamin A might be due to the difference in binding ability of different proteins or due to the adherence of the vitamin A on the walls of container. Lower binding ability of protein resulted in higher concentration of unbound vitamin A in solution during the preparation of milk protein-Vit A complexes. Unbound vitamin A may adhere on the wall of container (O'Brien and Robertson, 1993) and resulted in lower recovery of vitamin A from milk protein-Vit A complexes.

**Table 4.12: Recovery of vitamin A before and after lyophilisation of complexes**

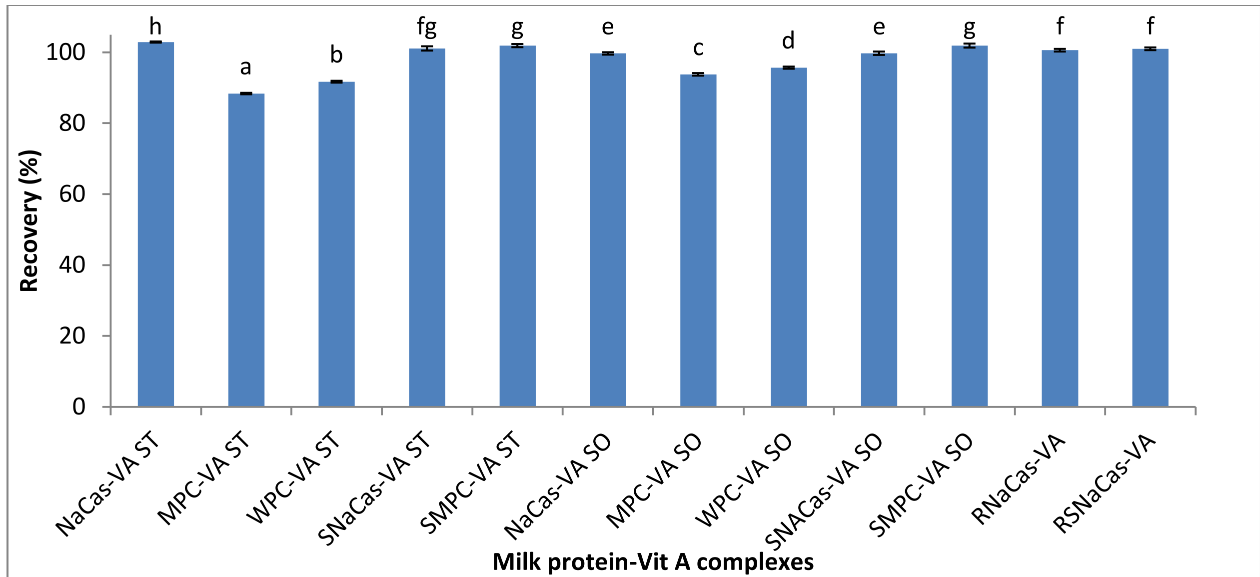
| Different complexes | Recovery (%) before lyophilisation | Recovery (%) after lyophilisation |
|---|---|---|
| NaCas-VA ST | 102.90±0.17$^{A}$ | 100.54±0.71$^{A}$ |
| MPC-VA ST | 88.37±0.21$^{A}$ | 87.79±0.50$^{A}$ |
| WPC-VA ST | 91.71±0.26$^{A}$ | 90.62±0.57$^{A}$ |
| SNaCas-VA ST | 101.10±0.61$^{A}$ | 100.95±0.26$^{A}$ |
| SMPC-VA ST | 101.90±0.45$^{A}$ | 99.24±0.84$^{A}$ |
| NaCas-VA SO | 99.69±0.35$^{A}$ | 98.57±0.36$^{A}$ |
| MPC-VA SO | 93.79±0.37$^{A}$ | 92.54±0.46$^{A}$ |
| WPC-VA SO | 95.67±0.31$^{A}$ | 94.73±0.58$^{A}$ |
| SNaCas-VA SO | 99.74±0.48$^{A}$ | 99.20±0.16$^{A}$ |
| SMPC-VA SO | 101.90±0.57$^{A}$ | 100.59±0.65$^{A}$ |
| RNaCas-VA | 100.60±0.37$^{A}$ | 99.63±0.43$^{A}$ |
| RSNaCas-VA | 101.00±0.39$^{A}$ | 100.60±0.49$^{A}$ |

Data are presented as means±SEM (n=3)
$^{A-B}$Means within rows with different superscript are significantly different ($p<0.05$) from each other.



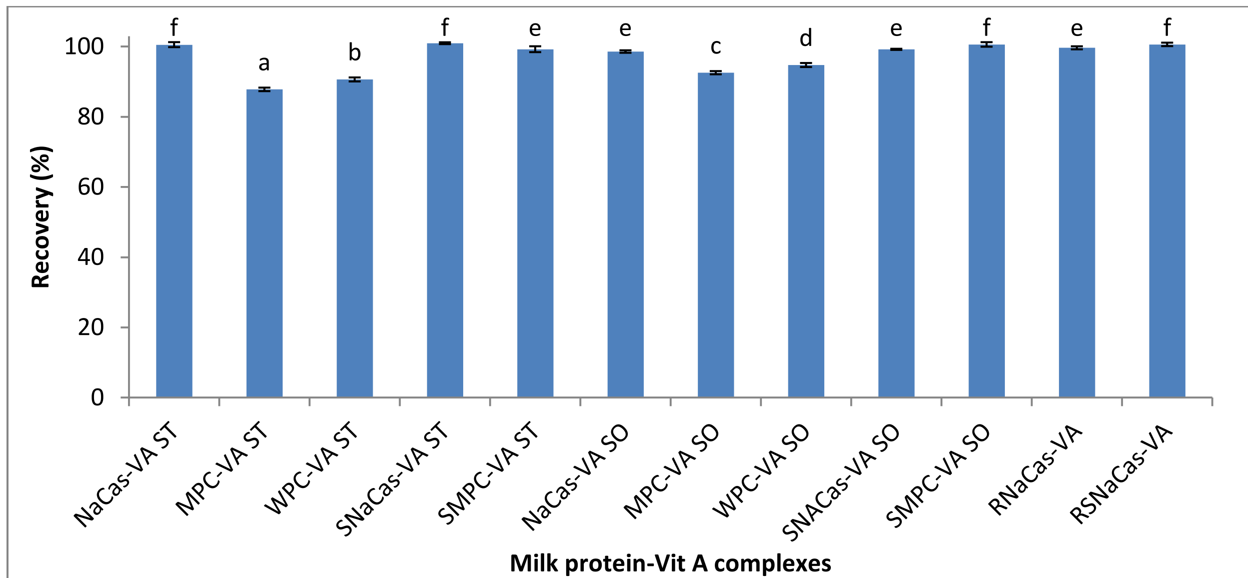

$^{A-B}$Samples represented with different letters are significantly different ($p<0.05$) from each other.
Error bars show the variations of three determinations in terms of standard error of mean.

**Fig 4.12: Comparison of recovery of vitamin A before and after lyophilisation of complexes**




[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.

**(a)**




[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.

**(b)**

**Fig 4.13: Recovery of vitamin A (a) before and (b) after lyophilisation of milk protein-Vit A complexes**

Zimet *et al.* (2011) studied the binding of DHA in reassembled casein micelles and found that only 62.3±0.5% of DHA was extracted from the complex after thermal

treatment (74°C for 20 s) and reported that 38% loss was due to imperfect extraction and losses during thermal treatment. Extraction of DHA from protein free suspension resulted in 93% recovery of DHA. Diarrassouba *et al.* (2015) reported that ß-lactoglobulin based coagulum can entrap vitamin $D_3$ with encapsulation efficiency (EE) of 94.5±1.8%. Luo *et al.* (2012) encapsulated vitamin $D_3$ in zein based nanoparticles with and without carboxymethyl cellulose and reported EE of 71.5% and 52.2%, respectively. Loading efficiency of alginate (hydrophobic derivative) based vitamin $D_3$ nanoparticles was 45.8-67.6% (Li *et al*., 2011). Zein based hydrogel beads can bind vitamin $D_3$ with EE of 96.9%, however, this preparation involved the use of glutaraldehyde as cross linking agent which is a toxic compound (Luo *et al*., 2013).

**4.7.2 Binding ability of milk proteins to bind vitamin A**

Milk protein samples were subjected to stirring, sonication and reassembling and their binding ability with vitamin A was evaluated. All milk protein-Vit A complexes showed significant difference ($p<0.05$) in the binding ability before and after lyophilisation of the samples (Table 4.13 and Fig 4.14). Dehydration of casein micelles using ethanol increased the hydrophobic interaction between the different casein molecules (Trejo and Harte, 2010). Increased hydrophobicity of protein due to dehydration might be responsible for the increased binding ability of vitamin A with proteins after lyophilisation.

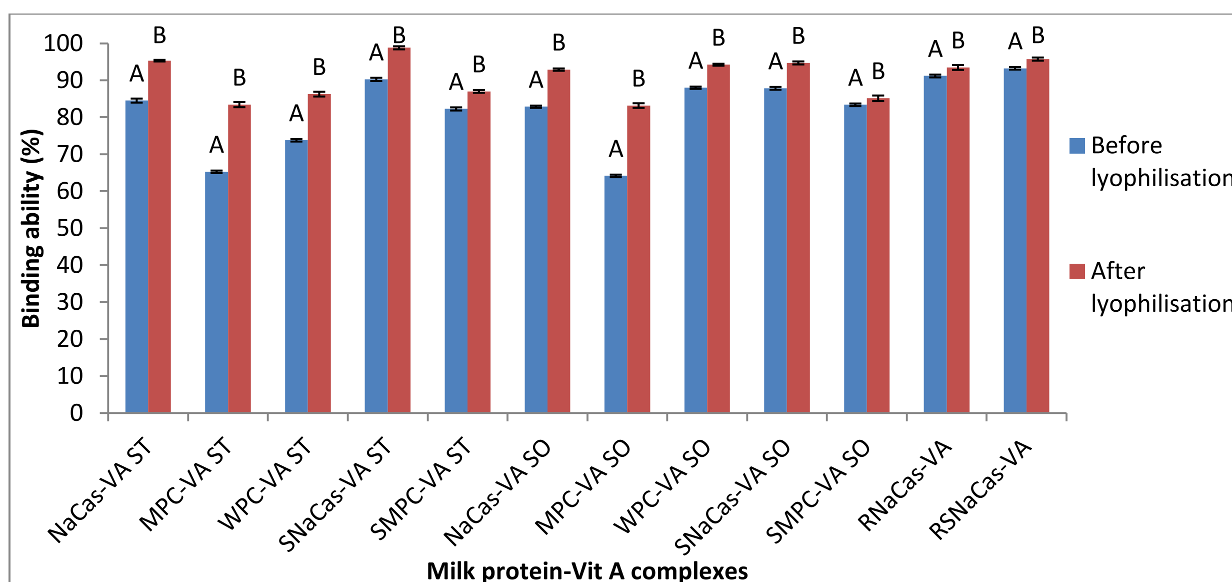
Reassembling resulted in highest binding ability as compared to stirred and sonicated samples both before and after lyophilisation of the samples (Fig 4.15 (a) and (b)). Increased binding ability of reassembled samples may be due to greater exposure of interior hydrophobic groups during disassembling and reassembling of casein micelles and vitamin A will bind with the interior hydrophobic groups. Haham *et al.* (2012) proposed that during reassembling vitamin D (VD) was incorporated into the hydrophobic core. Further, it was suggested that upon addition of VD (dissolved in ethanol) to caseinate solution, ethanol dissipated in water and self aggregation of VD occurred. Caseinate interfered in the self aggregation resulting in adsorption of VD on casein micelles. Semo *et al.* (2007) reported the use of casein micelles as natural nano-capsular vehicle for vitamin $D_2$. These vehicles can be used as a carrier of vitamin $D_2$ in dairy products without modifying the sensory properties. It was reported that 27.5% of

**Table 4.13: Binding ability of vitamin A before and after lyophilisation of milk protein-Vit A complexes**

| Different complexes | Binding ability (%) before lyophilisation | Binding ability (%) after lyophilisation |
|---|---|---|
| NaCas-VA ST | 84.52±0.55$^{A}$ | 95.31±0.21$^{B}$ |
| MPC-VA ST | 65.24±0.35$^{A}$ | 83.42±0.70$^{B}$ |
| WPC-VA ST | 73.77±0.33$^{A}$ | 86.26±0.64$^{B}$ |
| SNaCas-VA ST | 90.23±0.44$^{A}$ | 98.79±0.42$^{B}$ |
| SMPC-VA ST | 82.26±0.43$^{A}$ | 86.99±0.38$^{B}$ |
| NaCas-VA SO | 82.8500.31$^{A}$ | 92.89±0.32$^{B}$ |
| MPC-VA SO | 64.14±0.38$^{A}$ | 83.16±0.63$^{B}$ |
| WPC-VA SO | 87.99±0.32$^{A}$ | 94.21±0.26$^{B}$ |
| SNaCas-VA SO | 87.82±0.38$^{A}$ | 94.70±0.41$^{B}$ |
| SMPC-VA SO | 83.37±0.36$^{A}$ | 85.13±0.77$^{B}$ |
| RNaCas-VA | 91.17±0.38$^{A}$ | 93.47±0.67$^{B}$ |
| RSNaCas-VA | 93.24±0.34$^{A}$ | 95.74±0.43$^{B}$ |

Data are presented as means±SEM (n=3)

$^{A-B}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.




$^{A-B}$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.

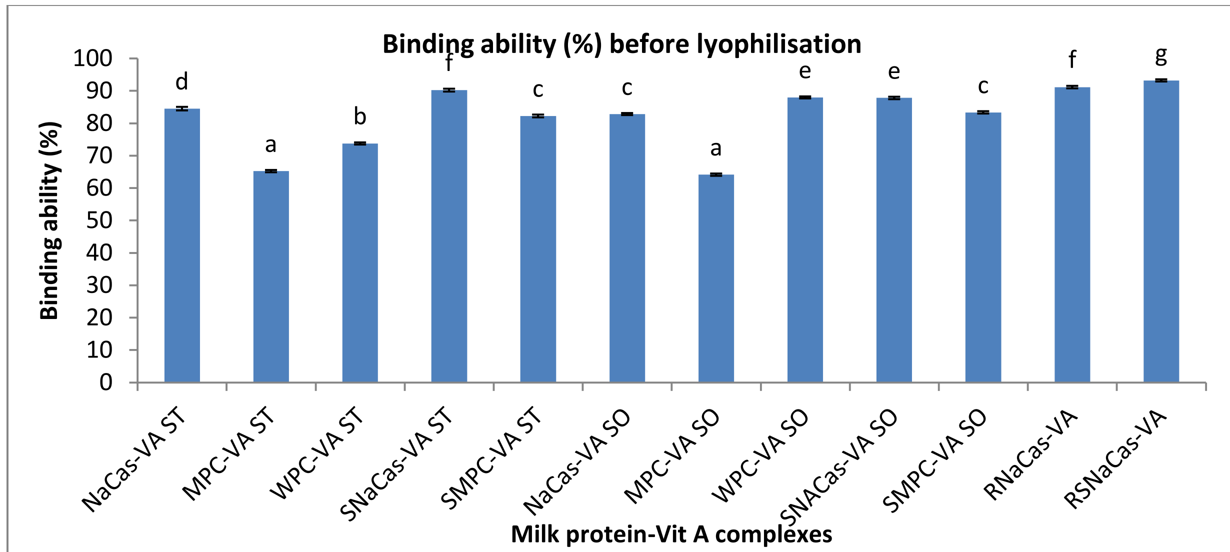
**Fig 4.14: Comparison of binding ability of vitamin A before and after lyophilisation of complexes**

vitamin $D_2$ was associated with the pellet, 72.5% with the serum and 0% in the ultrafiltration permeate.

Sonication resulted in significantly lower ($p<0.05$) binding ability as compared to stirring, however, sonication improved the binding ability of WPC both before and after lyophilisation of the samples (Fig 4.15 (a) and (b)). Casein lacks secondary and tertiary structures, hence, casein structure was slightly affected by sonication, however, whey proteins contained buried hydrophobic groups which were exposed on sonication and this resulted in higher binding ability of sonicated WPC-VA complexes. Fox and McSweeney (1998) reported that caseins have high surface hydrophobicity, in contrast to the globular whey proteins, in which the hydrophobic residues are buried as much as possible within the molecule, with most of the hydrophilic residues exposed on the surface. Caseins lack secondary and tertiary structures, hence the hydrophobic residues are rather exposed. O'Sullivan *et al.* (2014) reported that sonication treatment reduced the intrinsic viscosity and water binding ability which resulted in increased hydrophobicity of milk proteins. High intensity ultrasound treatment causes molecular unfolding of the WPC molecules and exposure of more hydrophobic groups and interior region of molecule to the more polar surrounding environment which resulted in increased surface hydrophobicity of the WPC (Arzeni *et al.* 2012). Sonication of BSA for 45 min at 20 $Wcm^{-2}$ causes unmasking of previously hidden hydrophobic group (Gulseren *et al.* 2007).

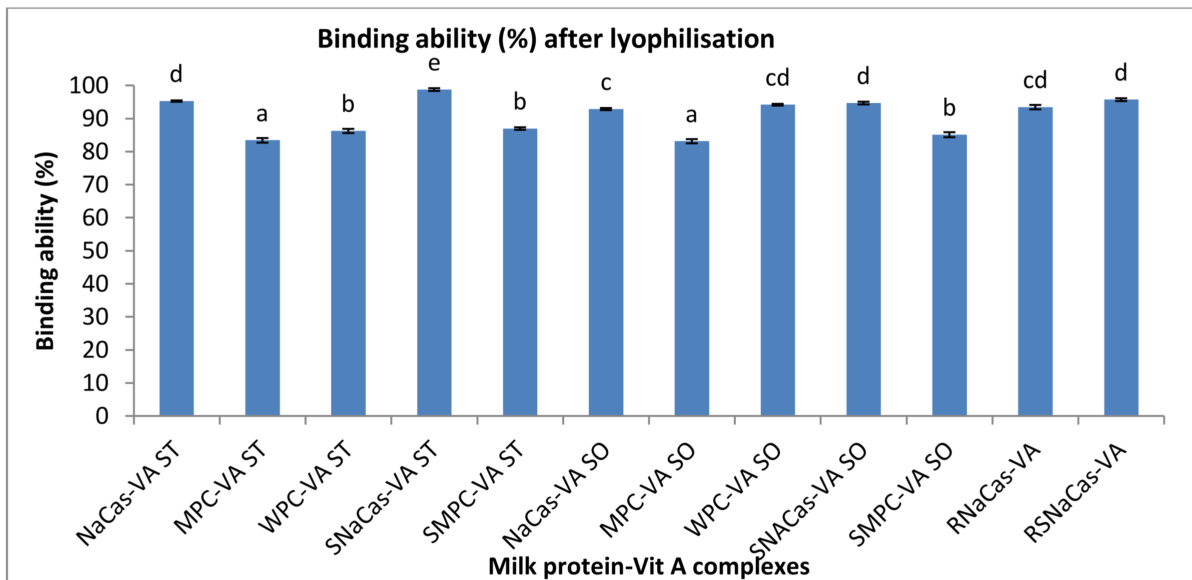
Chandrapala *et al.* (2012) reported that few minutes of sonication disrupted the casein-whey protein aggregation and resulted in the size reduction of casein micelles due to disruption of disulphide bond. Sonication resulted in lower binding ability of vitamin A with NaCas, SNaCas, MPC and SMPC due to disruption of hydrophobic bonds between milk protein and vitamin A. Ghasemi and Abbasi (2014) used casein micelles for encapsulation of fish oil and compared the effect of pH and ultrasonication on encapsulation efficiency. It was reported that ultrasonication for 4 min resulted in lower encapsulation efficiency of casein micelles as compared to the effect of pH.

Succinylated proteins (SNaCas, SMPC, RSNaCas) showed significantly higher ($p<0.05$) binding ability as compared to their native protein (NaCas, MPC, RNaCas) counterparts both before and after lyophilisation of the samples (Fig 4.15 (a) and (b)).




[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.

**(a)**




[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.

**(b)**

**Fig 4.15: Binding ability of vitamin A (a) before and (b) after lyophilisation of milk protein-Vit A complexes**

Succinylation resulted in extensive unfolding of protein molecules due to the high negative charge on the protein molecules (Thompson and Reyes 1980). Succinylation resulted in unaltered amino acid composition with only slight decrease in lysine content

of protein. Ser, Thr, Cys, Tyr and His can also be succinylated, although the reaction may be slower or the products are less stable than produced with Lys. Major amino acid involved in the interaction of protein with hydrophobic compound are Tyr, Phe, Trp, Leu and Val (Belatik *et al.* 2012; Bourassa *et al.* 2013; Forrest *et al.* 2005). Succinylation affect mainly Lys, other amino acid involved in hydrophobic interaction may not be affected, therefore, modified protein might be able to bind with hydrophobic compounds. Succinylation also increased the hydrophobicity of milk proteins due to the conformational changes which resulted in greater exposure of hydrophobic core. This might be due to increased electrostatic repulsion (Lakkis and Villota, 1992). Choi *et al.* (1981), El-Adawy (2000), Mirmoghtadaie *et al.* (2009), Monteiro and Prakash (1996), Poonampolam *et al.* (1988) and Sheen (1991) also reported the improved oil absorption capacity of various proteins on succinylation.

MPC-VA and SMPC-VA showed significantly lower ($p<0.05$) binding ability as compared to NaCas-VA and SNaCas-VA complexes both before and after lyophilisation of the samples (Fig 4.15 (a) and (b)). MPC showed lower binding ability due to the interference of the solute (lactose and whey protein) which creates hindrance in the interaction of vitamin A to protein (Yuksel *et al.* 2010).

Chevalier-Lucia *et al.* (2011) studied the binding of α-tocopherol to phosphocasein dispersion. Samples were centrifuged at 23160 g for 30 min at 20°C. Ultracentrifugation pellet (UCF-PC) contains the bound α-tocopherol. UCF-PC was able to bind 0.27 and 0.37 mol of α-tocopherol/mol of casein at 20°C and 37°C, respectively. Blayo *et al.* (2014) reported that pressure denatured whey protein and phosphocasein assemblies were able to bind 2.3-3.7 nmol and 3.8-5.4 nmol of retinyl acetate per mg of protein.

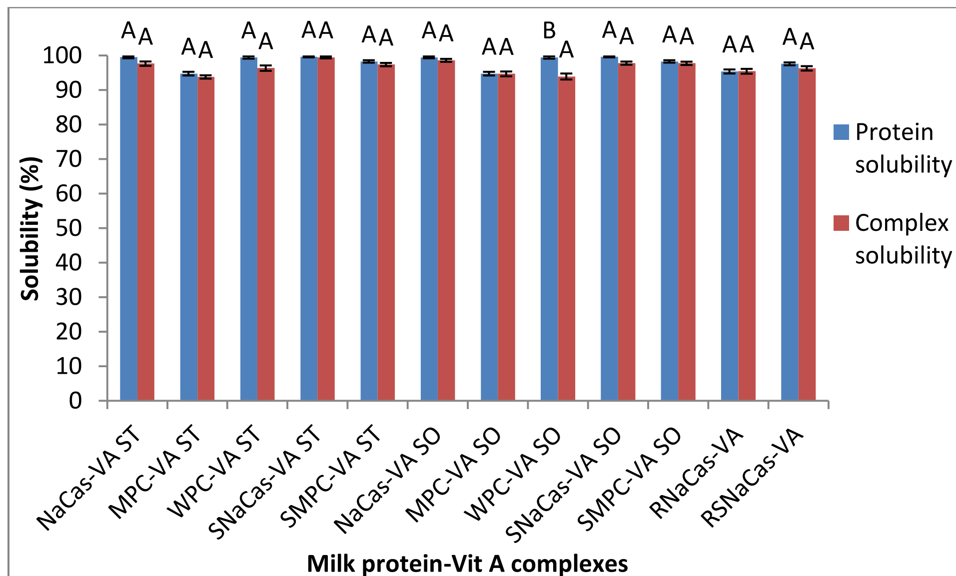
### 4.7.3 Solubility of protein as affected by binding of vitamin A

Solubility of protein before and after binding to vitamin A was compared. All milk protein samples showed non significant difference ($p>0.05$) in solubility of protein after complexation with vitamin A, except WPC-VA SO which showed significant decrease ($p<0.05$) in protein solubility (Table 4.14 and Fig 4.16). WPC-VA SO showed lower protein solubility, which might be due to the exposure of hydrophobic groups on sonication (Arzeni *et al.* 2012). RNaCas and RSNaCas showed significantly lower

**Table 4.14: Effect of milk protein-Vit A complex formation on protein solubility**

| Different complexes | Protein solubility (%) | Milk protein-Vit A complex solubility (%) |
|---|---|---|
| NaCas-VA ST | 99.48±0.29$^{A}$ | 97.63±0.63$^{A}$ |
| MPC-VA ST | 94.74±0.53$^{A}$ | 93.78±0.48$^{A}$ |
| WPC-VA ST | 99.43±0.36$^{A}$ | 96.34±0.78$^{A}$ |
| SNaCas-VA ST | 99.62±0.16$^{A}$ | 99.45±0.30$^{A}$ |
| SMPC-VA ST | 98.27±0.36$^{A}$ | 97.36±0.12$^{A}$ |
| NaCas-VA SO | 99.48±0.29$^{A}$ | 98.59±0.42$^{A}$ |
| MPC-VA SO | 94.74±0.53$^{A}$ | 94.67±0.70B$^{A}$ |
| WPC-VA SO | 99.43±0.36$^{B}$ | 93.91±0.87$^{A}$ |
| SNaCas-VA SO | 99.62±0.16$^{A}$ | 97.78±0.46$^{A}$ |
| SMPC-VA SO | 98.27±0.36$^{A}$ | 97.73±0.48$^{A}$ |
| RNaCas-VA | 95.37±0.59$^{A}$ | 95.42±0.70$^{A}$ |
| RSNaCas-VA | 97.58±0.42$^{A}$ | 96.27±0.64$^{A}$ |

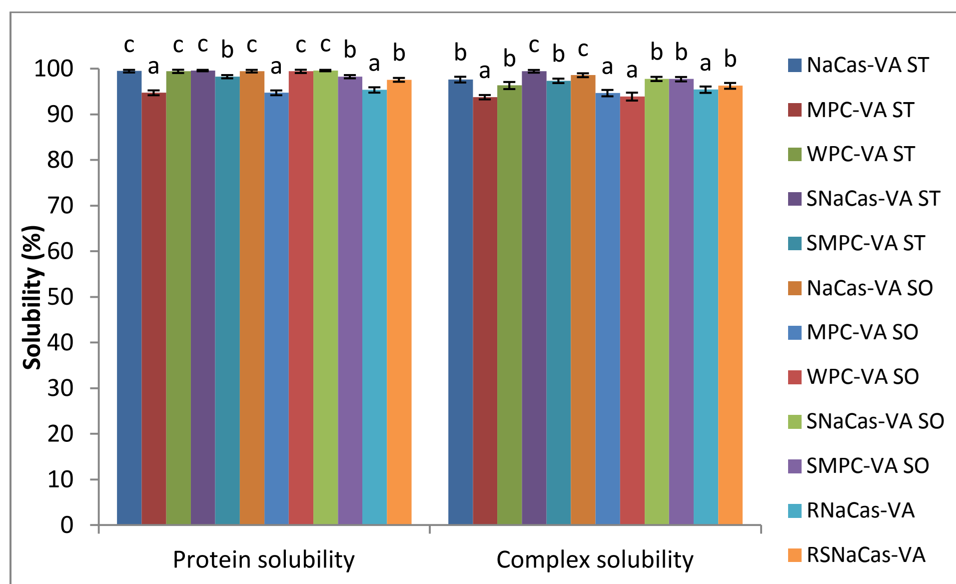
Data are presented as means±SEM (n=3)
$^{A-B}$Means within rows with different superscript are significantly different (p<0.05) from each other.




$^{A-B}$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.

**Fig 4.16: Effect of vitamin binding on milk protein solubility (%)**

(p<0.05) solubility as compared to NaCas and SNaCas, respectively (Fig 4.17). This may be due to the exposure of some hydrophobic groups during reassembling of the protein.




[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.

**Fig 4.17: Comparison of solubility (%) of different protein and different milk protein-Vit A complexes**

MPC showed lowest protein solubility, however succinylation improved the solubility of MPC. MPC-VA ST, MPC-VA SO and WPC-VA SO showed lower protein solubility, however, SNaCas-VA ST and NaCas-VA SO showed higher protein solubility. Other complexes showed intermediate values for protein solubility (Fig 4.17). WPC-VA ST showed significantly higher (p<0.05) protein solubility as compared to WPC-VA SO (Fig 4.17) due to the exposure of hydrophobic groups on sonication.

#### 4.7.4 Solubility of vitamin A as affected by complexation to milk protein

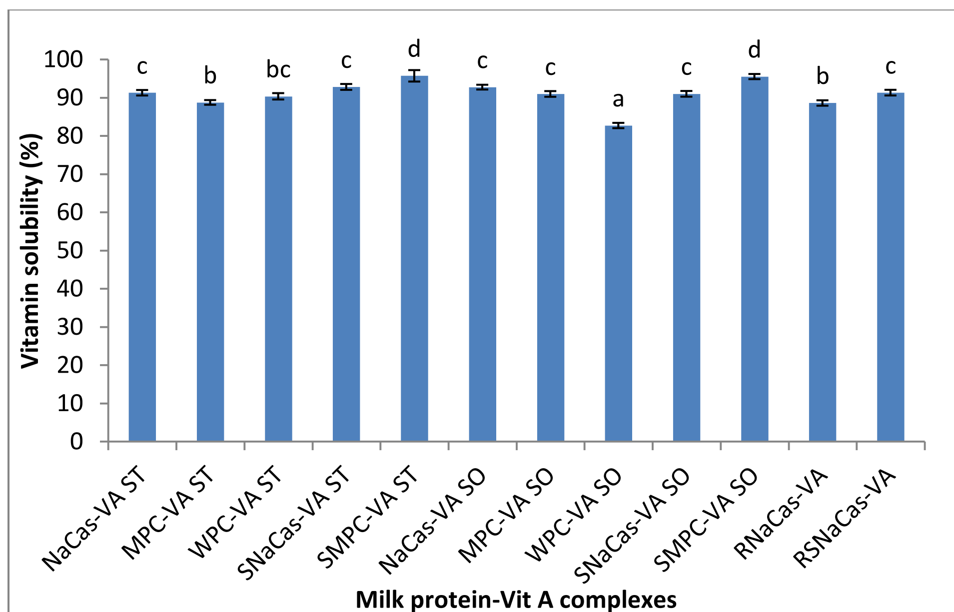
Solubility of vitamin A was associated with the solubility of milk protein-Vit A complexes. It was observed that, there was a positive correlation between solubility of milk protein-Vit A complexes and solubility of vitamin A (Table 4.15 and Fig 4.18).

Diarrassouba *et al.* (2015) reported that the solubility of fat soluble vitamin increased upon sequestering within the protein matrix. Liang *et al.* (2011) also reported that binding of α-tocopherol to ß-lg reduced the turbidity and improved the solubility of

**Table 4.15: Solubility (%) of vitamin A after preparation of milk protein-Vit A complexes**

| Different complexes | Vitamin A solubility (%) after complexation |
|---|---|
| NaCas-VA ST | 91.29±0.72$^{c}$ |
| MPC-VA ST | 88.76±0.61$^{b}$ |
| WPC-VA ST | 90.36±0.84$^{bc}$ |
| SNaCas-VA ST | 92.82±0.77$^{c}$ |
| SMPC-VA ST | 95.72±1.50$^{d}$ |
| NaCas-VA SO | 92.76±0.62$^{c}$ |
| MPC-VA SO | 90.98±0.74$^{c}$ |
| WPC-VA SO | 82.72±0.69$^{a}$ |
| SNaCas-VA SO | 91.01±0.75$^{c}$ |
| SMPC-VA SO | 95.53±0.67$^{d}$ |
| RNaCas-VA | 88.61±0.71$^{b}$ |
| RSNaCas-VA | 91.32±0.74$^{c}$ |

Data are presented as means±SEM (n=3)
$^{a-b}$Means within columns with different superscript are significantly different (p<0.05) from each other.




$^{a-b}$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.

**Fig 4.18: Solubility (%) of vitamin A after preparation of milk protein-Vit A complexes**

fat soluble vitamin. Binding of curcumin to ß-CN improved the solubility of curcumin (Esmaili *et al.* 2011). Ron *et al.* (2010) reported the binding of vitamin $D_2$ to ß-lactoglobulin pectin complex and found that complexation improved the solubility of vitamin $D_2$. It was also reported that these complexes can be used as nanovehicles for vitamin $D_2$ and hydrophobic nutraceuticals in non-fat foods and clear beverages.

### 4.7.5 Selection of milk protein-Vit A complexes from native and succinylated milk protein

One of the major objective of this research work was to select milk protein-Vit A complexes from both native and succinylated protein. Selection was based upon the ability of milk protein to bind with vitamin A and the solubility of milk protein-Vit A complex (Table 4.16).

**Table 4.16: Binding ability and solubility of milk protein-Vit A complexes**

| Different complexes | Binding ability (%) | Milk protein-Vit A complex solubility (%) |
|---|---|---|
| NaCas-VA ST | $95.31 \pm 0.21^{d}$ | $97.63 \pm 0.63^{b}$ |
| MPC-VA ST | $83.42 \pm 0.70^{a}$ | $93.78 \pm 0.48^{a}$ |
| WPC-VA ST | $86.26 \pm 0.64^{b}$ | $96.34 \pm 0.78^{b}$ |
| SNaCas-VA ST | $98.79 \pm 0.42^{e}$ | $99.45 \pm 0.30^{c}$ |
| SMPC-VA ST | $86.99 \pm 0.38^{b}$ | $97.36 \pm 0.12^{b}$ |
| NaCas-VA SO | $92.89 \pm 0.32^{c}$ | $98.59 \pm 0.42^{c}$ |
| MPC-VA SO | $83.16 \pm 0.63^{a}$ | $94.67 \pm 0.70^{a}$ |
| WPC-VA SO | $94.21 \pm 0.26^{cd}$ | $93.91 \pm 0.87^{a}$ |
| SNaCas-VA SO | $94.70 \pm 0.41^{d}$ | $97.78 \pm 0.46^{b}$ |
| SMPC-VA SO | $85.13 \pm 0.77^{b}$ | $97.73 \pm 0.48^{b}$ |
| RNaCas-VA | $93.47 \pm 0.67^{cd}$ | $95.42 \pm 0.70^{a}$ |
| RSNaCas-VA | $95.74 \pm 0.43^{d}$ | $96.27 \pm 0.64^{b}$ |

Data are presented as means±SEM (n=3)
$^{a-b}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.

NaCas-VA ST, SNaCas-VA ST, RNaCas-VA, RSNaCas-VA and WPC-VA SO showed higher binding ability as compared to the other complexes. NaCas-VA ST,

SNaCas-VA ST, WPC-VA ST, SMPC-VA ST, NaCas-VA SO, SNaCas-VA SO, SMPC-VA SO, RNaCas-VA and RSNaCas-VA showed higher solubility as compared to the other complexes. WPC-VA ST, SMPC-VA ST, NaCas-VA SO, SNaCas-VA SO and SMPC-VA SO showed lower binding ability and WPC-VA SO showed lower solubility. On the basis of above results NaCas-VA ST, SNaCas-VA ST, RNaCas-VA and RSNaCas- VA were selected for further studies.

## 4.8 Optimization of concentration of vitamin A bound to milk protein-Vit A complexes

During the selection of milk protein-Vit A complexes, 10000 IU of vitamin A was bound to 5 gm of milk protein. Among all milk protein-Vit A complexes, RNaCas-VA complex showed lowest binding ability, hence, it was selected for the optimization of vitamin A concentration that can be bound to milk protein-Vit A complexes with minimum affect on binding ability. During optimization of concentration of vitamin A bound to milk protein-Vit A complexes, vitamin A concentration was increased from 10000 IU to 5.0 lakh IU/5 gm of protein. Similar concentration of vitamin A should bind to same amount of protein for comparison of vitamin A stability in different milk protein-Vit A complexes, hence only one complex was evaluated for optimization of concentration of vitamin A.

**Table 4.17: Effect of concentration on binding ability (%) of RNaCas-VA complexes**

| Concentration of Vitamin A (IU) (bound to 5 gm protein) | Binding ability (%) |
|---:|:---:|
| 10000 IU | 95.28±0.17 |
| 40000 IU | 90.14±0.34 |
| 100000 IU | 90.19±0.48 |
| 200000 IU | 90.79±0.44 |
| 300000 IU | 87.71±0.47 |
| 350000 IU | 83.67±0.40 |
| 400000 IU | 82.45±0.46 |
| 450000 IU | 81.87±0.42 |
| 500000 IU | 69.91±0.44 |

Sharp decrease in binding ability of RNaCas-VA complexes was observed after 4.5 lakh IU/5 gm protein (Table 4.17 and Fig 4.19), hence, this concentration of vitamin

A was selected for the further studies. NaCas-VA ST, SNaCas-VA ST, RNaCas-VA and RSNaCas-VA complexes were prepared using the optimized vitamin A concentration and evaluated for physicochemical characterization, stability and *in-vitro* bioavailability of vitamin A.

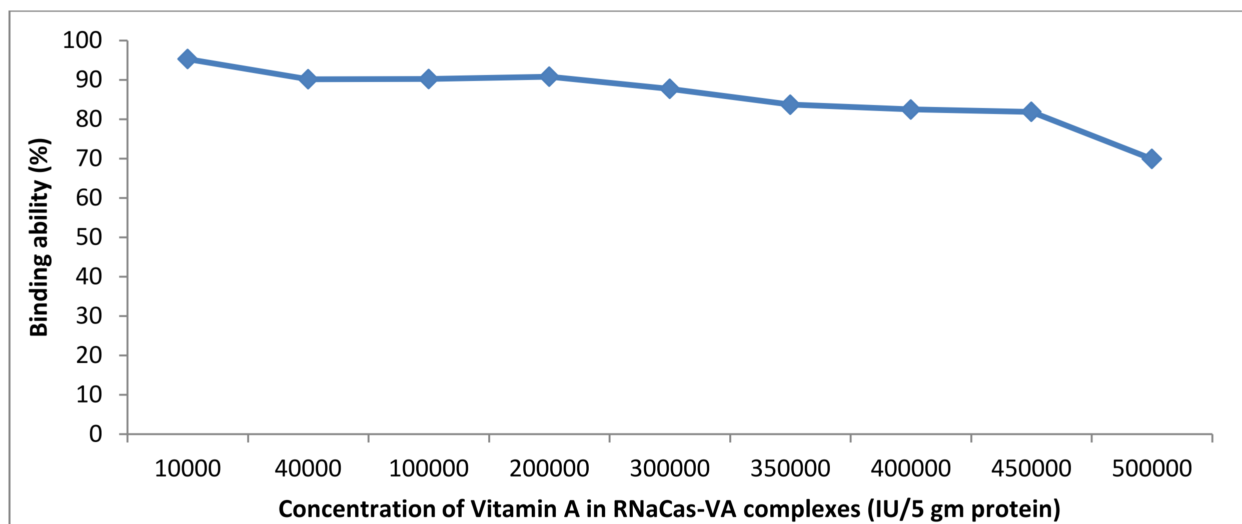



**Fig 4.19: Effect of concentration of vitamin A on binding ability (%) of RNaCas-VA complexes**

## 4.9 Physicochemical characterization, stability and *in-vitro* bioavailability of milk protein-Vit A complexes

### 4.9.1 Physicochemical characterization

Selected complexes were analysed for physicochemical characteristics as discussed below.

#### 4.9.1.1 Vitamin A and protein content in milk protein-Vit A complexes

NaCas-VA ST showed highest vitamin and protein content followed by SNaCas-VA ST, RNaCas-VA and RSNaCas-VA complexes (Table 4.18). Protein content in NaCas and SNaCas was 90% and 85%, respectively (Table 4.5). During reassembling tri potassium citrate, $CaCl_2$ and $K_2HPO_4$ were retained with the protein which resulted in overall decrease in the protein content of reassembled protein.

Binding of vitamin A to protein further decreased the overall protein content of milk protein-Vit A complexes. Due to the decrease in protein content, higher quantity of

protein powder was used for the preparation of milk protein-Vit A complex which further resulted in decrease in vitamin A content per 100 mg of complex.

**Table 4.18: Vitamin A and protein content in milk protein-Vit A complexes**

| Milk protein-Vit A Complexes | Vitamin A content (IU/100 mg of complex) | Protein content (%) |
|---|---|---|
| **NaCas-VA ST** | 10400.08±262.78 | 87.44±0.27 |
| **SNaCas-VA ST** | 8455.23±300.49 | 81.19±0.18 |
| **RNaCas-VA** | 6828.94±167.93 | 69.66±0.16 |
| **RSNaCas-VA** | 6450.13±262.77 | 62.19±0.53 |

### 4.9.1.2 Turbidity analysis

Vitamin A, milk protein and milk protein-Vit A complexes were dissolved in double distilled water and turbidity of the samples was analysed as % transmittance. Turbidity of milk protein-Vit A complex solution was due to both milk protein and vitamin A, hence, turbidity caused by vitamin A (10000 IU/10 ml) was estimated after deduction of turbidity of milk protein solution from turbidity of milk protein-Vit A complex solution. In case of milk protein-Vit A complex, vitamin A contributes to the major part of turbidity and it was observed that binding of vitamin A to different milk proteins resulted in significant reduction ($p<0.05$) in turbidity caused by the vitamin A. Vitamin A bound to RNaCas showed lowest turbidity followed by RSNaCas, NaCas, SNaCas and free

**Table 4.19: Turbidity of vitamin A solution as affected by milk protein-Vit A complexation**

| Samples | Turbidity | Turbidity due to vitamin A (10000 IU/10 ml) |
|---|---|---|
| NaCas | 13.33±0.09 | |
| NaCas-VA ST | 92.72±0.14 | 79.39±0.23$^{c}$ |
| SNaCas | 7.07±0.19 | |
| SNaCas-VA ST | 89.64±0.35 | 82.57±0.54$^{d}$ |
| RNaCas | 4.10±0.14 | |
| RNaCas-VA | 60.08±0.26 | 55.97±0.12$^{a}$ |
| RSNaCas | 10.56±0.18 | |
| RSNACas-VA | 71.92±0.23 | 61.36±0.05$^{b}$ |
| VA | 89.19±0.19 | 89.19±0.19$^{e}$ |

Data are presented as means±SEM (n=3)

$^{a-b}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.

vitamin A (oily form) (Table 4.19). Reassembled milk protein-Vit A complexes showed lower turbidity may be due to the binding of vitamin A to the hydrophobic core. Haham *et al.* (2012) also reported the binding of vitamin $D_3$ in the hydrophobic core of reassembled casein micelles.

Addition of ß-lactoglobulin to α-tocopherol solution resulted in reduced turbidity due to the formation of more hydro-soluble complex (Liang *et al.* 2011). Fat soluble vitamins are prone to self aggregation which resulted in increase in size and thus increase in turbidity (Dubbs and Gupta, 1998; Haham *et al.* 2012; Liang *et al.* 2011). Binding of vitamin A to milk protein reduced the turbidity which might be due to the reduction in self aggregation of vitamin A and formation of more hydrosoluble complex.

**4.9.1.3 Particle size and zeta (ζ) potential**

Milk protein-Vit A complexes showed significantly higher ($p<0.05$) particle size and ζ-potential as compared to milk proteins. Succinylation of NaCas resulted in decrease in particle size and increase in ζ-potential, however, reassembling of NaCas and SNaCas resulted in increase in particle size and decrease in ζ-potential (Table 4.20, Fig 4.20, 4.21 and 4.22).

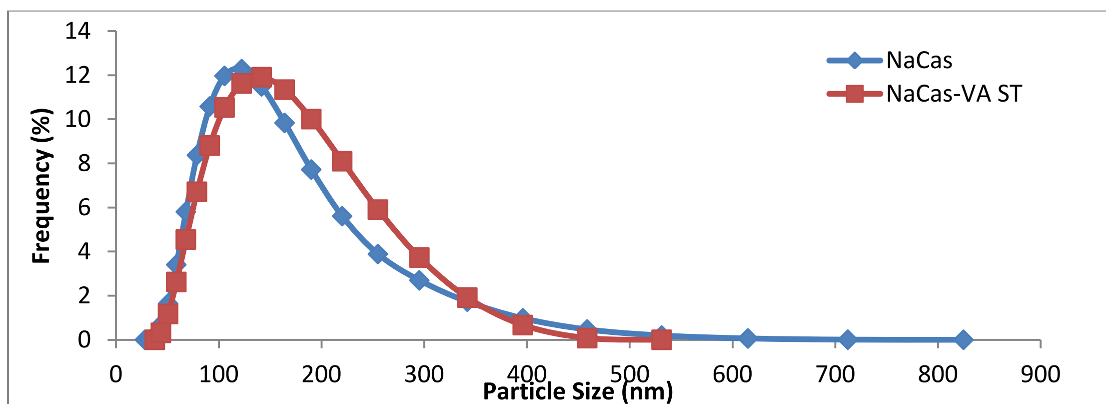
Relkin and Shukat (2012) reported that the presence of α-tocopherol in WPC dispersion resulted in increased backscattering of light which indicated an increase in particle size due to vitamin-protein interaction. Addition of α-tocopherol to protein dispersion enhanced ζ-potential values by approx -10mV. Increase in particle size and surface charge were due to the possible protein swelling mechanism resulting from increased repulsive forces between amino acid located in the core of the protein molecules, which may be due to vitamin entrapment in the protein core or between protein aggregrates. Zimet *et al.* (2011) reported that DHA loaded casein nanoparticles (CNP) showed larger diameter or particle size as compared to pure casein and DHA loaded reassembled casein micelles (r-CM). DHA loaded r-CM were prepared at 4°C, however, DHA loaded CNP were prepared at 37°C. Hydrophobic interactions were lower at low temperature; hence, casein-casein, casein-DHA and DHA-DHA interactions were weaker which resulted in smaller aggregrates and nanoparticles. Haham *et al.* (2012) and Semo *et al.* (2007) reported that binding of vitamin $D_3$ and vitamin $D_2$ to r-CM (156 nm) increases the size of r-CM (147nm). Ron *et al.* (2010) reported that

**Table 4.20: Evaluation of the effect of vitamin A binding on particle size (nm) and ζ-potential (mV) of milk protein**

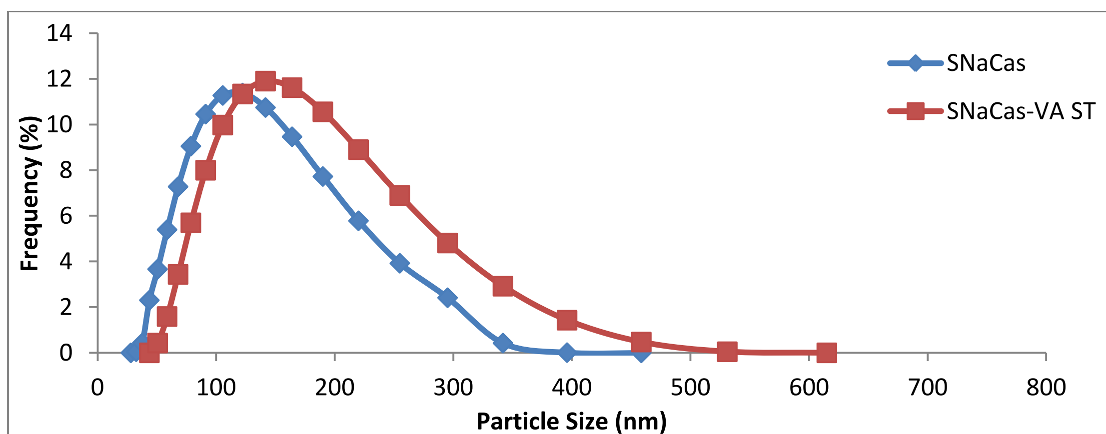
| Samples | Particle size (nm) | ζ-potential(mV) |
|---|---|---|
| NaCas | $123.57 \pm 1.11^{a}$ | $-22.33 \pm 0.67^{c}$ |
| NaCas-VA ST | $142.12 \pm 4.32^{b}$ | $-24.3 \pm 0.06^{cd}$ |
| SNaCas | $115.07 \pm 3.54^{a}$ | $-27.50 \pm 1.10^{d}$ |
| SNaCas-VA ST | $135.13 \pm 3.45^{ab}$ | $-31.73 \pm 0.27^{e}$ |
| RNaCas | $154.27 \pm 7.66^{bc}$ | $-11.70 \pm 0.35^{a}$ |
| RNaCas-VA | $175.30 \pm 5.98^{cd}$ | $-19.00 \pm 0.12^{b}$ |
| RSNaCas | $168.27 \pm 5.04^{c}$ | $-16.40 \pm 0.77^{b}$ |
| RSNaCas-VA | $181.97 \pm 9.25^{d}$ | $-27.80 \pm 0.12^{d}$ |

Data are presented as means±SEM (n=3)

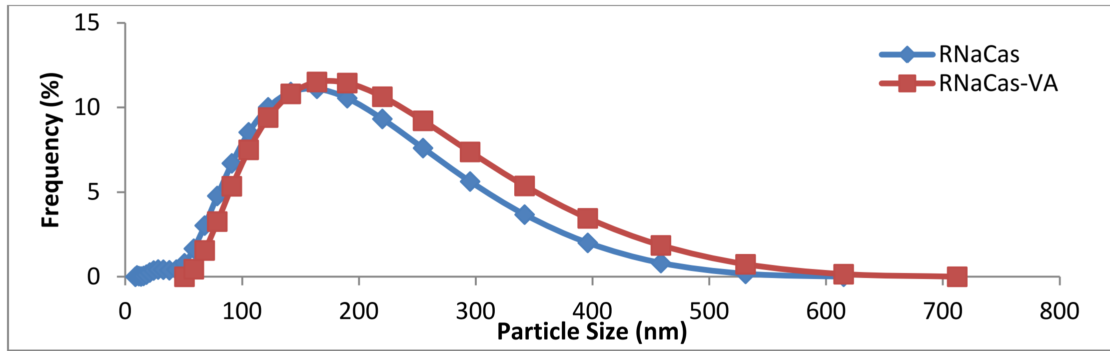
$^{a-b}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.



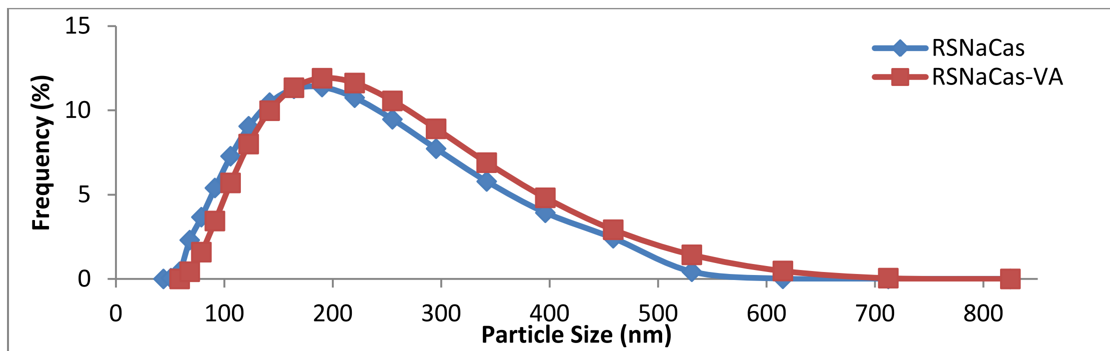

**(a)**




**(b)**

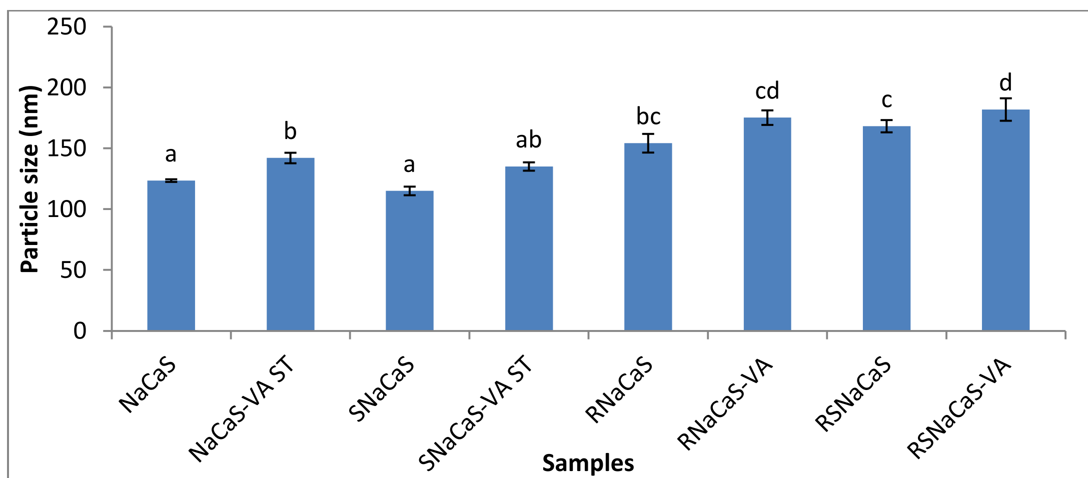



**(c)**



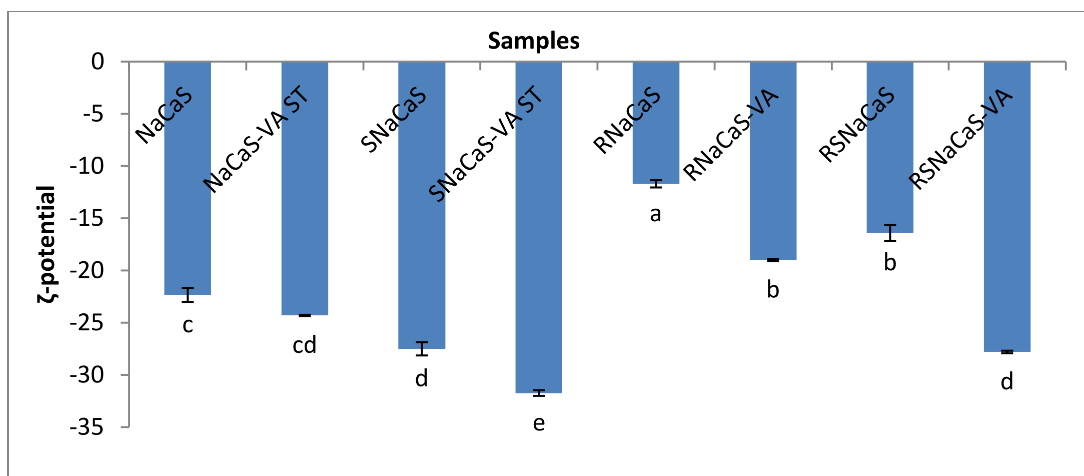

**(d)**

**Fig 4.20: Particle size distribution of milk protein and milk protein-Vit A complexes (a) NaCas and NaCas-VA ST, (b) SNaCas and SNaCas-VA ST, (c) RNaCas and RNaCas-VA and (d) RSNaCas and RSNaCas-VA**




[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.

**Fig 4.21: Evaluation of the effect of vitamin A binding on particle size (nm) of milk protein**




[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.

**Fig 4.22: Evaluation of the effect of vitamin A binding on ζ-potential (mV) of milk protein**

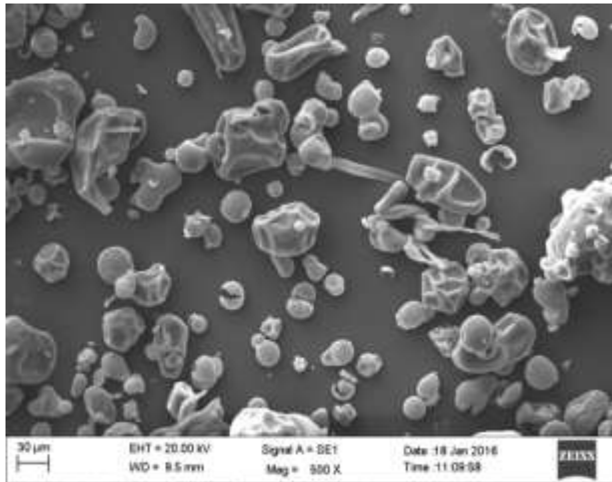
vitamin $D_2$ loaded ß-lactoglobulin-pectin nanoparticles (~56 nm) showed larger diameter as compared to ß-lactoglobulin-pectin complexes (35 nm). It was suggested that vitamin $D_2$ addition induces protein-protein aggregation which increases size of nanoparticles. Entrapment of vitamin $D_3$ in nanoparticles of whey protein isolate increased the size of particles (Abbasi *et al.* 2014). Blayo *et al.* (2014) studied the binding of retinyl acetate with phoshocasein dispersion and found non significant influence of retinyl acetate binding on particle size of complex. Perez *et al.* (2014a) reported that binding of folic acid to ß-lactoglobulin increased the size of complex. These complexes were stabilized by hydrophobic interaction, van der-Waals interaction or steric overlap interaction. Binding of folic acid to ß-lactoglobulin induced conformational changes resulting in exposing more charged area to protein surface, thus increasing electrophoretic mobility and ζ-potential. McClements (2005) reported that electrostatically stabilized hydrocollids commonly posses ζ-potential exceeding absolute values of 40 mV.

Shilpashree *et al.* (2015a, b) reported that succinylation of sodium caseinate and milk protein concentrate decreased the particle size and ζ-potential. Succinylation prevented the formation of aggregates which increased the solubility of succinylated milk proteins. Nakai and Li-chan (1989) reported that succinylation involved the replacement of attractive forces in native molecule with repulsive force, which prevented the formation of aggregates and hence, decreased particle size of native molecule. Zayas (1997)

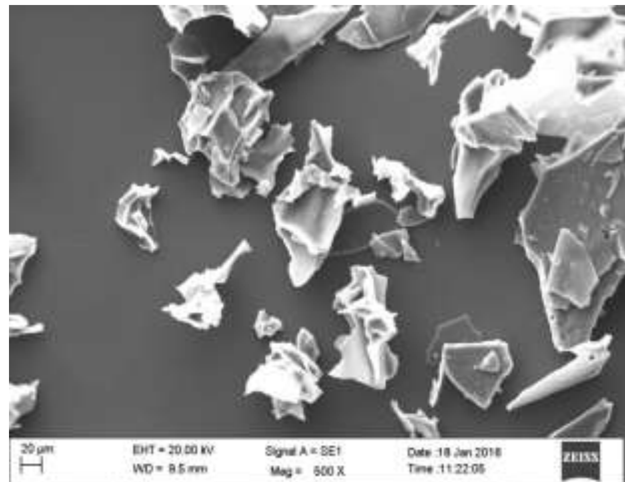
reported that succinylation increased the molecular surface area of the protein which resulted in electrical repulsion between identical charges and thus increased ζ-potential of succinylated protein.

### 4.9.1.4 Microstructural analysis by scanning electron microscopy

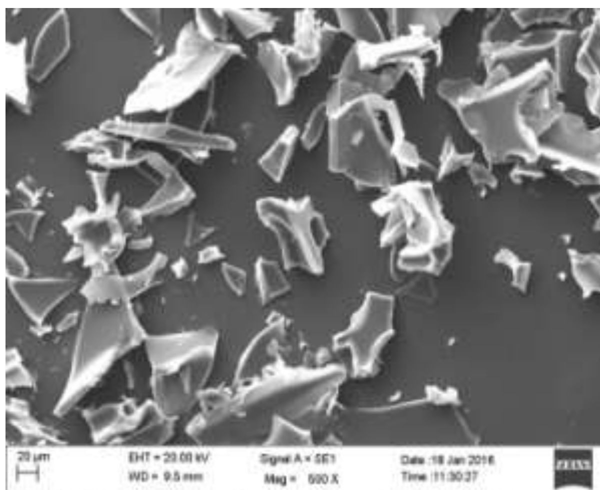
Microstructure details of milk protein-Vit A complexes as analysed by scanning electron microscope are presented in figure 4.23 (a-h). Micrograph of NaCas showed uniform spherical structure with some dents on the surface. Since, NaCas sample used was spray dried powder, it appeared as a circular particle. During spray drying, water gets evaporated and particles shrink, resulting in dents on the surface of circular particles. SNaCas, RNaCas, RSNaCas, NaCas-VA ST, SNaCas-VA ST, RNaCas-VA and RSNaCas-VA complexes were freeze dried which resulted in broken glass and



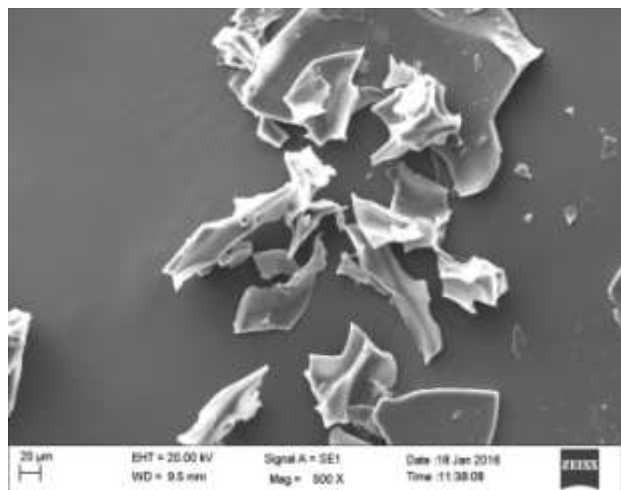

(a)




(b)




(c)




(d)

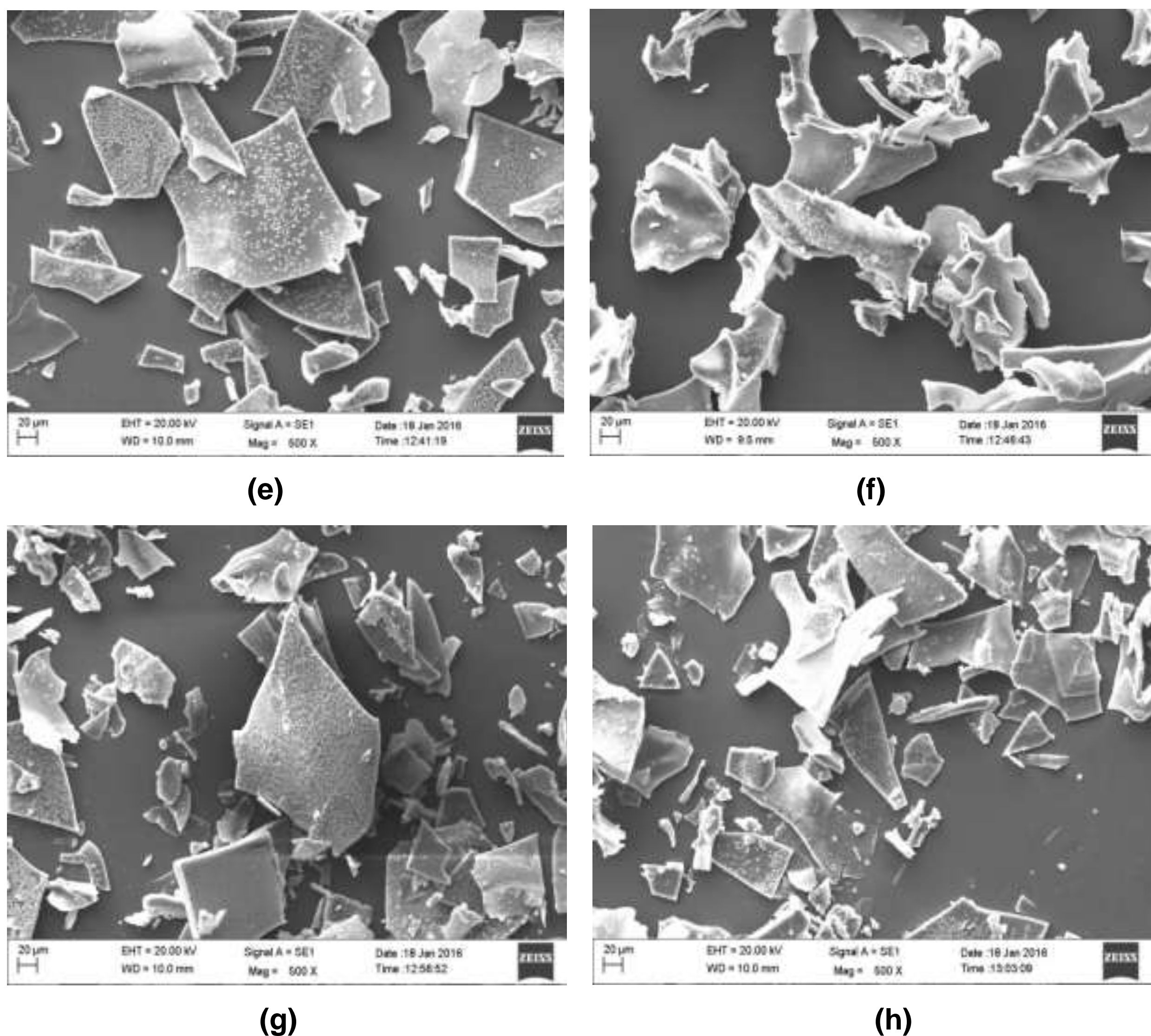



**Fig 4.23: Microstructural analysis by scanning electron microscopy (500 X) of milk protein-Vit A complexes (a) NaCas, (b) NaCas-VA ST, (c) SNaCas, (d) SNaCas-VA ST, (e) RNaCas, (f) RNaCas-VA, (g) RSNaCas and (h) RSNaCas-VA**

flaky structure. Low temperature during freeze drying resulted in breaking up of frozen liquid into droplets which altered their surface topology during the evaporation process and causes flake structure of freeze dried samples (Chen *et al.* 2012; Anandharamakrishnan *et al.* 2010). White tiny particles were observed on the surfaces of reassembled protein and reassembled protein-Vit A complexes. Saiz-Abajo *et al.* (2013) reported the similar morphology of ß-carotene-reassembled casein micelles

complex. Miller *et al.* (2012) reported the formation of flat plate-shaped brushite, dicalcium phosphate dihydrate ($CaHPO_4 \cdot 2H_2O$) crystal due to the reaction of $CaCl_2$ and alkaline phosphate salt solutions. These dicalcium phosphate crystals might be responsible for the formation of crystalline structures on the surface of reassembled protein and reassembled protein-Vit A complexes.

#### 4.9.1.5 Evaluation of the effect of vitamin binding on the mobility of protein

SDS-PAGE was performed to evaluate the modification in electrophoretic mobility of milk protein-Vit A complexes. 2.5 mg of each protein sample was dissolved per ml of sample buffer and further resolved on 14% separating gel. Figure 4.24 (a) depicted the mobility of low molecular weight markers and casein standards. κ-CN showed the highest mobility followed by ß-CN and α-CN. Figure 4.24 (b) and (c) depict the electrophoretic mobility of milk protein and milk protein-Vit A complexes. Electrophoretic pattern of milk protein was not affected by the binding of vitamin A i.e. there was no difference in the mobility of different protein fractions due to vitamin A binding. Succinylated protein and reassembled succinylated protein showed lower mobility as compared to their respective native proteins (NaCas) due to increase in the molecular weight of succinylated protein. Reassembled protein showed no difference in the electrophoretic mobility as compared to native protein (NaCas).

Bhawana (2012) reported that vitamin A and iron fortification does not have significant influence on electrophoretic mobility of milk proteins. Cremonesi and Caramazza (1993) reported that succinylation modified the electrophoretic behavior of both α-CN and ß-CN.

#### 4.9.1.6 Evaluation of the effect of vitamin binding on Tryptophan fluorescence intensities

**Intrinsic tryptophan intensity:**

Intrinsic tryptophan intensities of different milk proteins and milk protein-Vit A complexes are presented in figure 4.25. Binding of vitamin A to milk protein resulted in decrease in the tryptophan intensities and shift in emission peaks to higher wavelength. Fluorescence intensity of NaCas, SNaCas, RNaCas and RSNaCas was observed to be

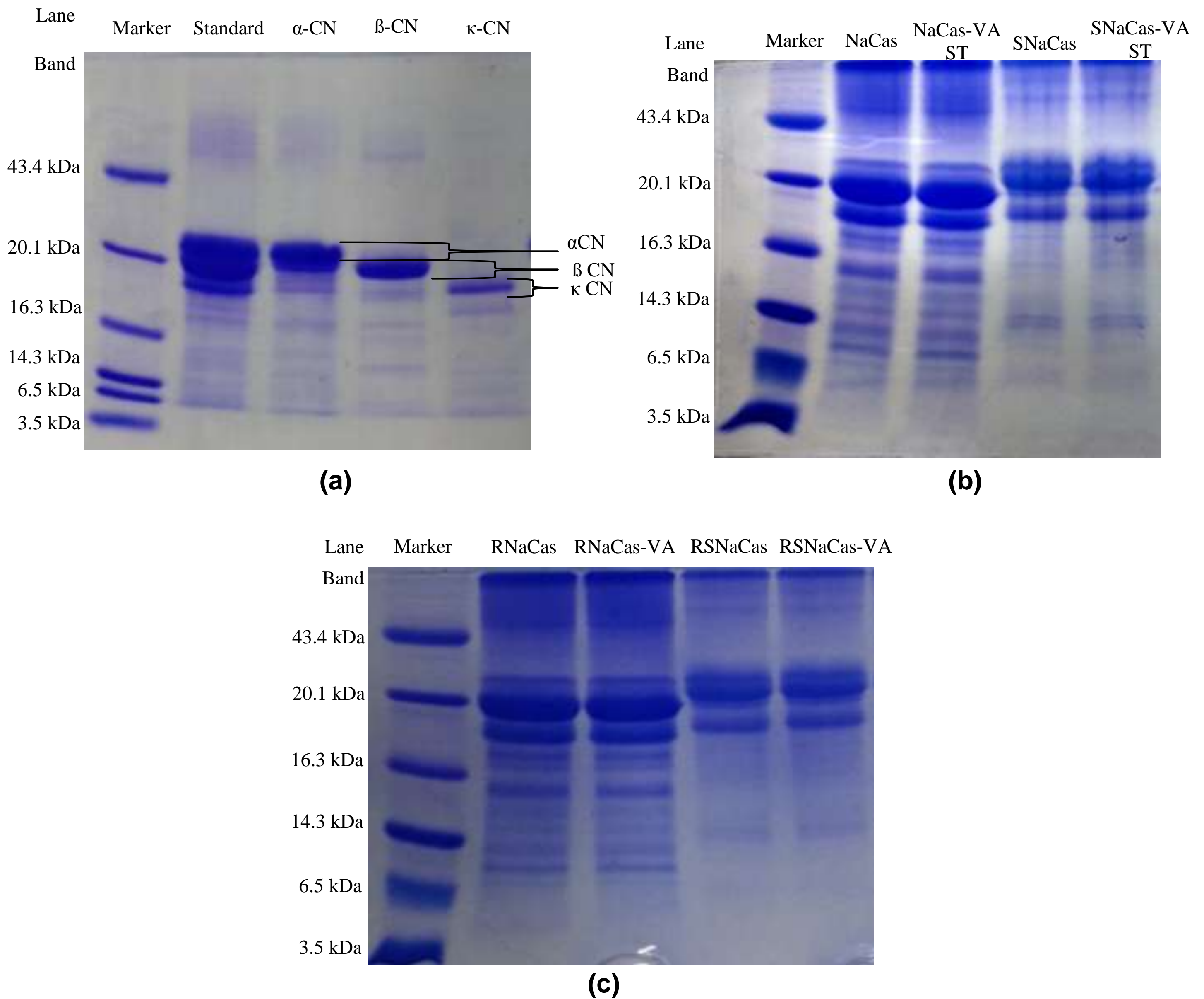
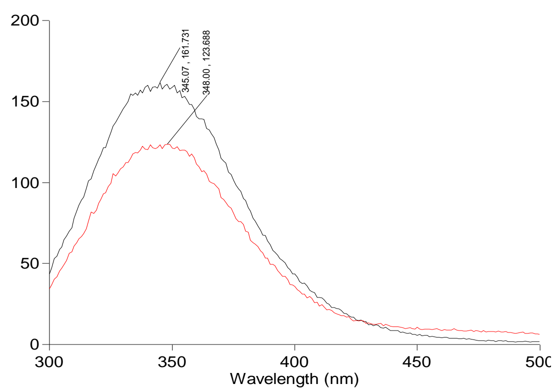
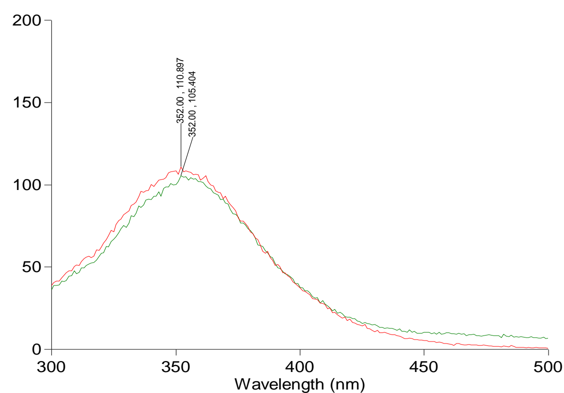


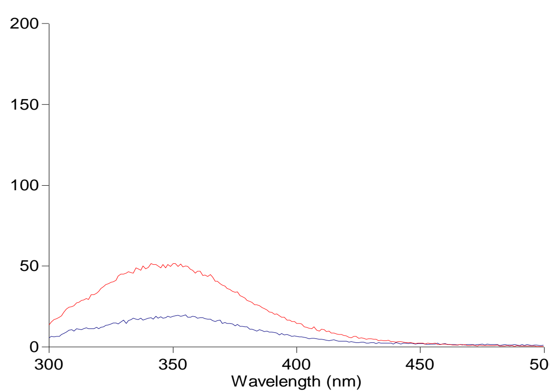

**Fig 4.24: SDS-PAGE pattern of (a) casein standards, (b) and (c) milk protein-Vit A complexes**

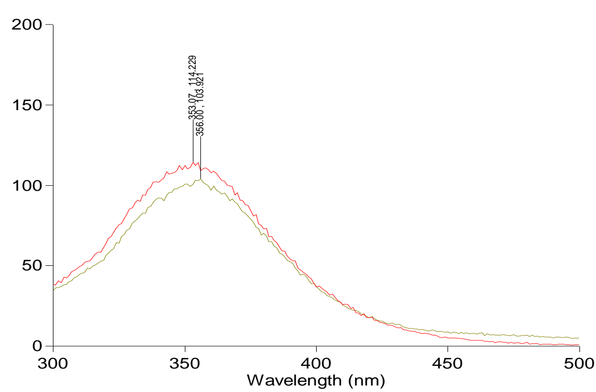
76.48%, 95.05%, 40% and 90.97% of the original intensity after binding with vitamin A (Fig 4.25 (a-d)). Binding of vitamin A to NaCas, SNaCas, RNaCas and RSNaCas resulted in 2.93 nm, 0 nm, 0nm and 2.93 nm red shift in the fluorescence peak, respectively. This shift in the peak indicated an increase in hydrophobicity of NaCas and RSNaCas after binding to vitamin A. The lower spectral shift observed may be due to the inherent molecular flexibity of casein (Lakkis and Villota 1992). Succinylation and reaasembling resulted in decrease in fluorescence intensity which indicated modification



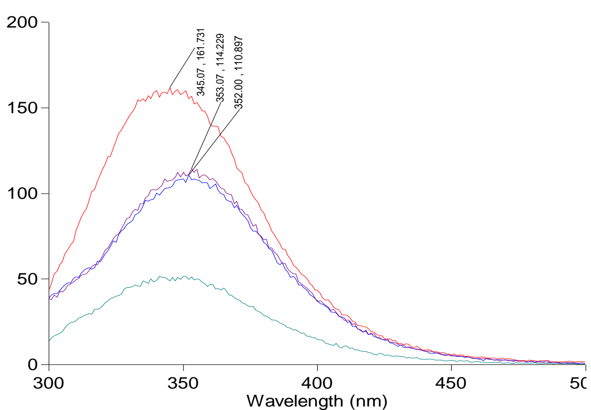



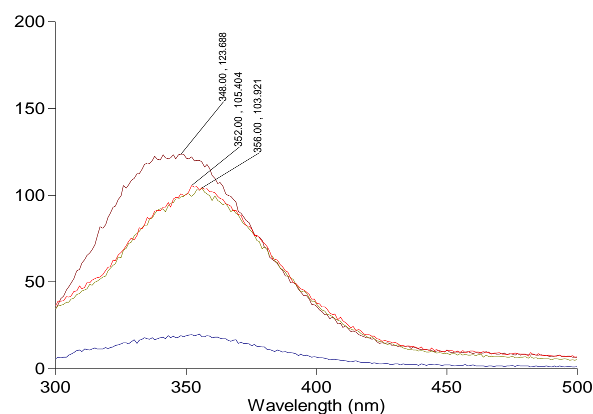

**(a)** **(b)**




**(c)** **(d)**




**(e)** **(f)**

**Fig 4.25: Intrinsic fluorescence spectra of (a) NaCas and NaCas-VA ST (upper to lower) (b) SNaCas and SNaCas-VA ST (upper to lower), (c) RNaCas and RNaCas-VA (upper to lower), (d) RSNaCas and RSNaCas-VA (upper to lower), (e) NaCas, RSNaCas, SNaCas and RNacas (upper to lower) and (f) NaCas-VA ST, SNaCas-VA ST, RSNaCas-VA and RNacas-VA (upper to lower)**

in protein structure. Succinylation and reassembling of NaCas reduced the fluorescence intensity to 68.56% and 30.91%, respectively. Reassembling of SNaCas does not have significant effect on fluorescence intensity, however, it showed significant reduction in fluorescence intensity as compared to native NaCas. RNaCas showed the minimum fluorescence intensity which indicated maximum modification in the protein structure during reassembling. Succinylation of NaCas and reassembling of SNaCas shifted the emission peaks towards longer wavelengths which indicated higher hydrobhobicity of protein. Succinylation of NaCas and reassembling SNaCas resulted in fluorescence peak shift of 6.93 nm and 8 nm, respectively (Fig 4.25 (e)).

Figure 4.25 (f) represents the comparison intrinsic fluorescence spectra of different milk protein-Vit A complexes. NaCas-VA ST showed the highest fluorescence intensity followed by SNaCas-VA ST, RSNaCas-VA and RNaCas-VA. SNaCas-VA ST, RNaCas-VA and RSNaCas-VA showed fluorescence intensity 85.21%, 16.17% and 84.02% as compared to NaCas-VA ST complexes (Table 4.21).

Red shift in emission peak is an indication of enhanced exposure of indole chromophore group of protein to the aqueous environment accompanied with some conformational changes and molecular unfolding. According to Lakkis and Villota (1992) higher red shift in fluorescence profile indicates higher degree of modification and subsequent enhancement of protein surface hydrophobicity.

**Table 4.21: Intrinsic tryptophan intensity of milk protein-Vit A complexes**

| Milk protein-Vit A Complexes | Intrinsic tryptophan intensity (%) |
|---|---|
| NaCas-VA ST | 100 |
| SNaCas-VA ST | 85.21 |
| RNaCas-VA | 16.17 |
| RSNaCas-VA | 84.02 |

Yazdi and Corredig (2012) reported that changes in the emission spectra of tryptophan is indicative of structural changes of proteins, as these changes affect the local environment that surrounds the indole ring, causing shifts in wavelength of maximum fluorescence and fluorescence intensity. Excited state of indole ring can donate electron to neighbouring molecules resulting in quenching of maximum

fluorescence intensity. They also reported that curcumin can penetrate to the inner core of casein micelles, which resulted in complete quenching of fluorescence signals. Curcumin also binds to the hydrophobic moieties of caseins present in close proximity of the calcium phosphate nanoclusters. Royer (1995) also reported that tryptophan fluorescence intensity may be used as a diagnostic tool for the conformational changes in folded protein structure as tryptophan has much stronger fluorescence and higher quantum yield than other aromatic amino acid (tyrosine and phenylalanine). Ellis *et al.* (2013), Vivian and Callis (2001) also reported that any variation in fluorescence intensities along with shift in emission peak to higher wavelength were true measure of change in the three dimensional structure of casein. Wavelength of emission peak of tryptophan if <330 nm, it is present in nonpolar environment and if above 330 nm it is present in polar environment. In case of milk, it is generally observed to be 342-343 nm, hence tryptophan is located in polar environment, due to open structure of casein micelles. Esmaili *et al.* (2011) reported the quenching of intrinsic fluorescence of ß-CN after binding to curcumin. Binding of α-tocopherol to WPC also resulted in decrease in fluorescence intensity (Relkin and Shukat 2012). Liang *et al.* (2011) reported that increase in concentration of α-tocopherol resulted in decreased fluorescence intensity of ß-lactoglobulin. Benzaria *et al.* (2013) reported that binding of curcumin to phosphocasein dispersion resulted in quenching of fluorescence intensity.

**Hydrophobicity**

ANS titration curve of milk protein and milk protein-Vit A complexes are presented in figure 4.26. 1% solution (on the basis of protein content) of milk protein and milk protein-Vit A complexes were prepared in double distilled water and used for ANS titration. Kinetic data from ANS titration curve were evaluated for relative maximum fluorescence ($F_{max}$ i.e. maximum fluorescence that could be attained under the given conditions and also the maximum number of surface sites allowable to which ANS could bound) and dissociation constant ($K_d$ i.e. ANS concentration required to obtain one half of the value of $F_{max}$). $1/K_d$ represent the binding affinity of ANS to milk protein, increase in $1/K_d$ indicates the increase in binding affinity. PSH is the protein surface hydrophobicity index ($H_o$) which measures the average tightness of fluorescence marker per protein.

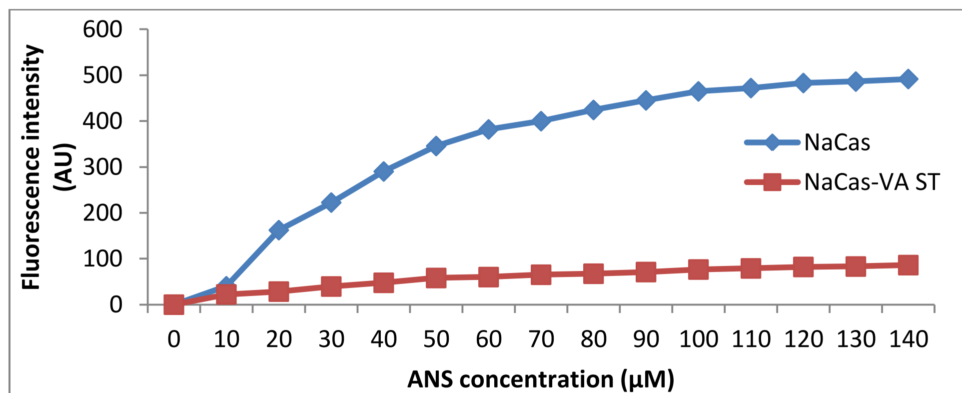
$$PSH=F_{max}/K_d*P$$

Where, P= Protein concentration (1%)

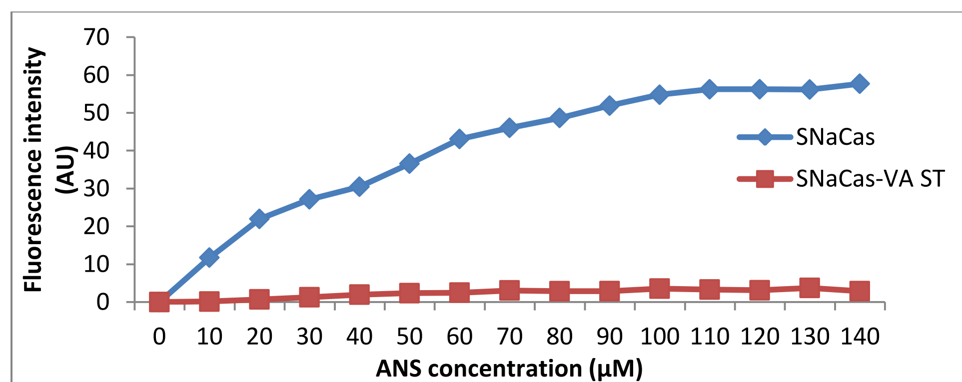
(Guo *et al.* 1996; Yuksel *et al.* 2010).

ANS binds to the protein hydrophobically at two hydrophobic sites (weak and strong) present on the surface of protein. Free form of ANS is not fluorescent in the solution, binding of ANS to the protein resulted in its fluorescent character at given excitation and emission wavelengths (Yuksel *et al.* 2010). Binding of ANS to protein is proportional to the fluorescence intensity of ANS. Higher the binding, higher will be the fluorescence intensity. Milk protein-Vit A complexes showed lower fluorescence intensity as compared to milk protein.

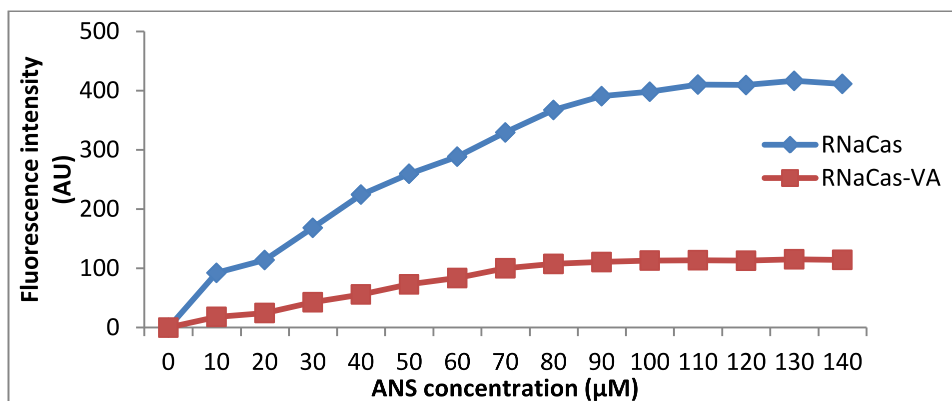
Vitamin A and ANS bind milk protein hydrophobically. Binding of vitamin A to milk protein blocks the available sites for interaction of ANS to milk protein, hence, resulting in decreased fluorescence of milk protein-Vit A complexes (Fig 4.26(a-d)). Yuksel *et al.* (2010) also reported that binding of green tea (GT) flavanoids prevented the binding of ANS to casein micelles due to possible interaction between casein and GT flavanoids.



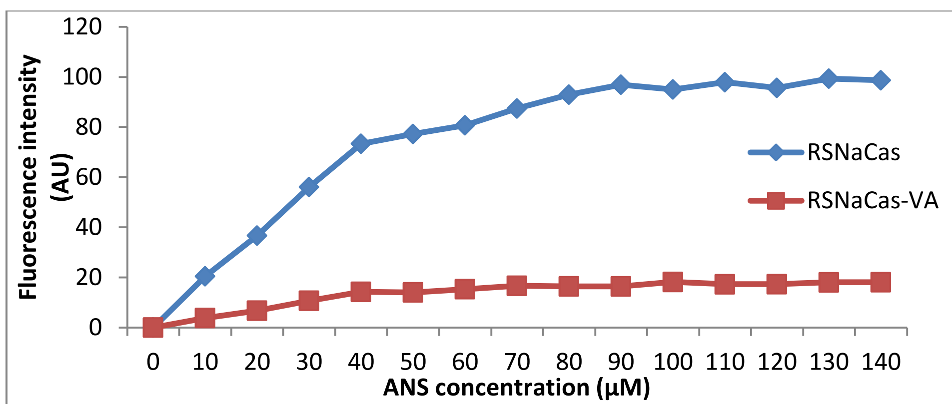

**(a)**




**(b)**




**(c)**




**(d)**

**Fig 4.26: ANS titration curves of milk protein and milk protein-Vit A complexes (a) NaCas, NaCas-VA ST, (b) SNaCas, SNaCas-VA ST, (c) RNaCas, RNaCas-VA and (d) RSNaCas, RSNaCas-VA**

Kinetic data obtained from ANS titration curves are presented in Table 4.22 and Fig 4.27. NaCas has the highest $F_{max}$ value followed by RNaCas, RSNaCas and SNaCas. Succinylation of NaCas resulted in decrease of $F_{max}$ values which indicate decreased hydrophobicity of SNaCas. Intrinsic tryptophan intensity and RP-HPLC profile of SNaCas showed increase in the hydrophobicity of NaCas after succinylation. Reduction in $F_{max}$ might be due to reduced binding of ANS to SNaCas. SNaCas had high electronegative charge on the surface and ANS is an anionic fluorescent dye (Matulis *et al.* 1999), which may resulted in lower interaction of ANS to SNaCas, hence reduction in $F_{max}$ was observed. Achouri and Zhang (2001) reported a significant decrease in protein surface hydrophobicity due to succinylation of soy protein hydrolysate. Succinylation increased the charge frequency and electronegativity which

in turn decreased the surface hydrophobicity due to hiding of hydrophobic core to ANS probe. Shilpashree *et al.* (2015a, b) reported that succinylation of NaCas modified the protein structure and presence of high negative charge on the surface reduced the penetration of ANS to bind ligands, hence, reduction in $F_{max}$ and $K_d$ values, as compared to native NaCas. Krishnan and Balaram (1976) also reported that the increase in negative charge of phospholipid created a shield over tryptophan residues and reduced the efficiency of anionic ANS probe to bind the ligands present in plasma proteins. Schwenke (1996) also reported that introduction of succinyl group decreases the hydrophobicity of pea legume. Kinsella (1989), Thompson and Reyes (1980) also reported that tyrosine also participates in succinylation along with lysine. Succinylation of tyrosine (ligand for ANS binding) may contribute to the reduction in $F_{max}$ upon succinylation.

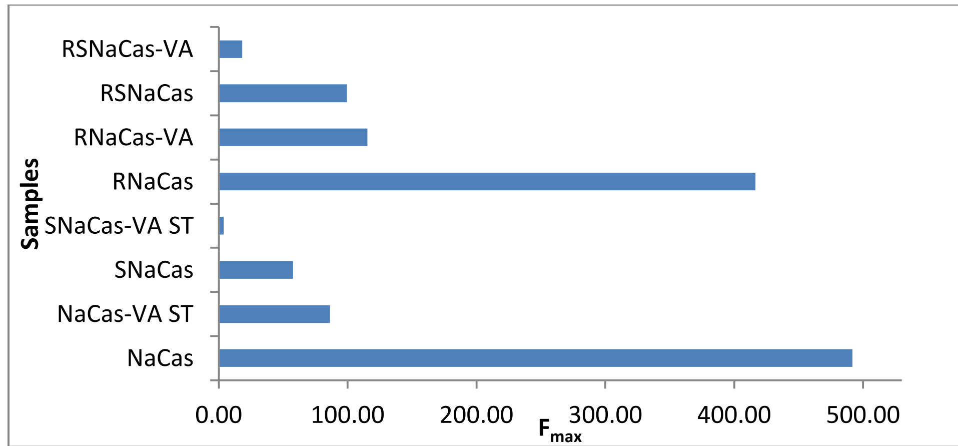
Reassembling of NaCas and SNaCas resulted in modification of protein structure. $F_{max}$ and PSH decreased for RNaCas but increased slightly for RSNaCas as compared to NaCas and SNaCas, respectively. Changes in $F_{max}$ and PSH were due to the modification in the structure of NaCas and SNaCas. During reassembling some hydrophobic groups may be buried inside which result in decrease of $F_{max}$ and PSH for RNaCas. In case of RSNaCas some groups with negative charge may also be buried inside alongwith hydrophobic groups which resulted in increased $F_{max}$ and PSH as compared to SNaCas. No literature is available regarding the effect of reassembling on the intrinsic tryptophan intensity and kinetic data of ANS titration curve.

Binding of vitamin A to milk protein decreased $F_{max}$ due to reduction in the surface sites available for binding with ANS. $1/K_d$ indicate the binding affinity of ANS to milk protein. During reassembling of NaCas, hydrophobic groups were buried inside the protein structure which resulted in decrease of $1/K_d$ value. Succinylation of NaCas and reassembling of SNaCas resulted in increase of $1/K_d$ value which indicated higher affinity of ANS to these proteins. Our results were in accordance with Shilpashree *et al.* (2015a, b) who reported increase in $1/K_d$ value upon succinylation of sodium caseinate and milk protein concentrate. Binding of vitamin A to milk protein resulted in decrease of $1/K_d$ value, which indicated the lower affinity of ANS to milk protein-Vit A complexes. $F_{max}/K_d$ or PSH indicate tightness or strength of binding (Yuksel *et al.* 2010).

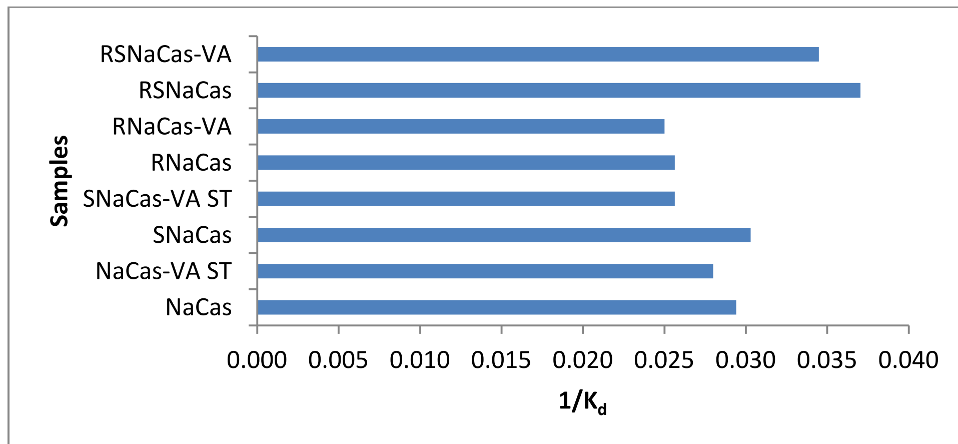
**Table 4.22: Kinetic data from ANS titration curve**

| Samples | $F_{max}$ | $1/K_d$ | PSH =($F_{max}/K_d$) |
|---|---|---|---|
| **NaCas** | 491.83 | 0.029 | 14.47 |
| **NaCas-VA** | 86.24 | 0.028 | 2.46 |
| **SNaCas** | 57.70 | 0.030 | 1.75 |
| **SNaCas-VA** | 3.74 | 0.026 | 0.10 |
| **RNaCas** | 416.51 | 0.026 | 10.68 |
| **RNaCas-VA** | 115.35 | 0.025 | 2.88 |
| **RSNaCas** | 99.33 | 0.037 | 3.68 |
| **RSNaCas-VA** | 18.09 | 0.034 | 0.62 |

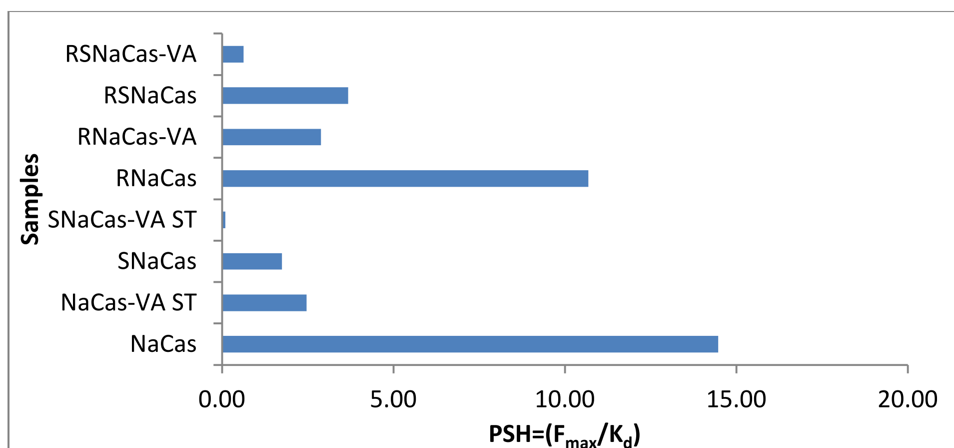
Succinylation of NaCas showed decrease in $F_{max}/K_d$ value or PSH as compared to NaCas, due to the presence of high electronegative charge on the surface. Binding of vitamin A to milk protein showed decrease in $F_{max}/K_d$ value or PSH, may be due to the interference of vitamin A during binding of ANS to milk protein.




**(a)**




**(b)**




**(c)**

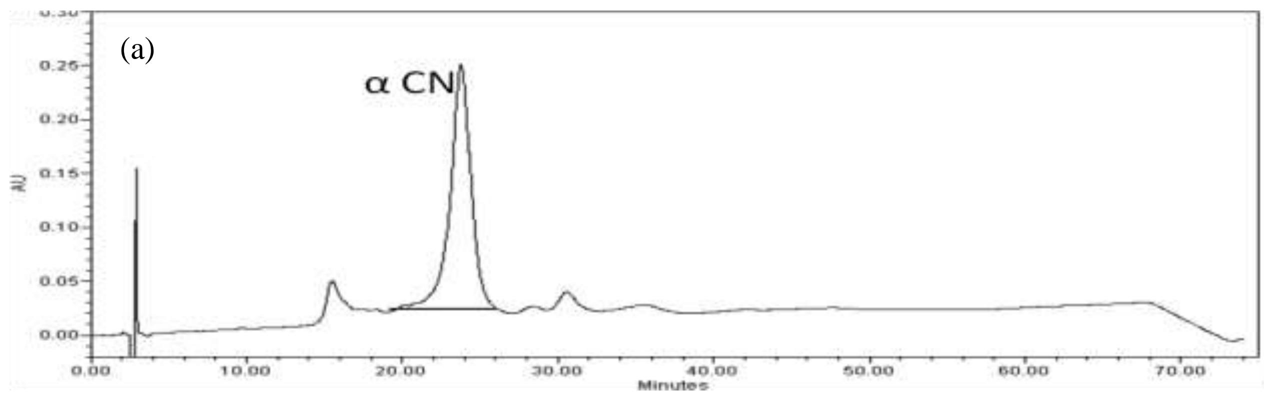
**Fig 4.27: (a) Number of surface hydrophobic sites (Fmax), (b) ANS binding affinity to the protein (1/Kd) and (c) Protein surface hydrophobicity ($H_o$) of milk protein and milk protein-Vit A complexes**

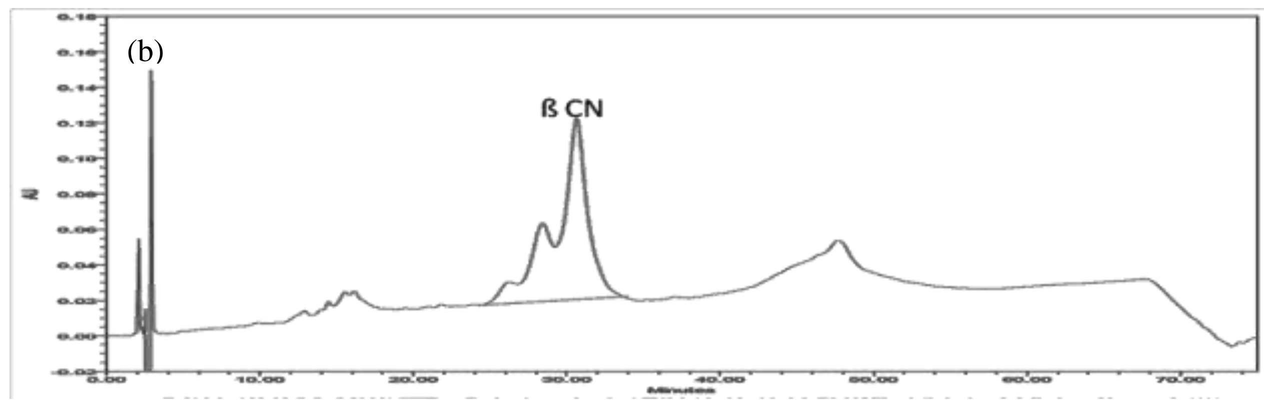
Yuksel *et al.* (2010) also reported that GT flavanoids prevented the binding of ANS to casein micelles due to the interaction between casein and GT flavanoids, which resulted in decrease of $F_{max}$ values, 1/Kd and PSH.

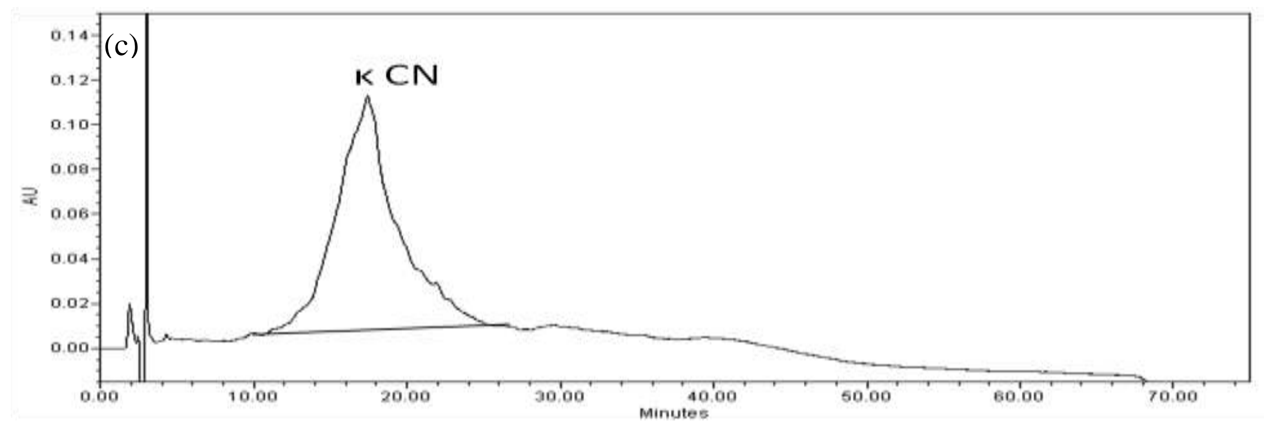
#### 4.9.1.7 Evaluation of the effect of vitamin binding on the elution characteristic of protein by RP-HPLC

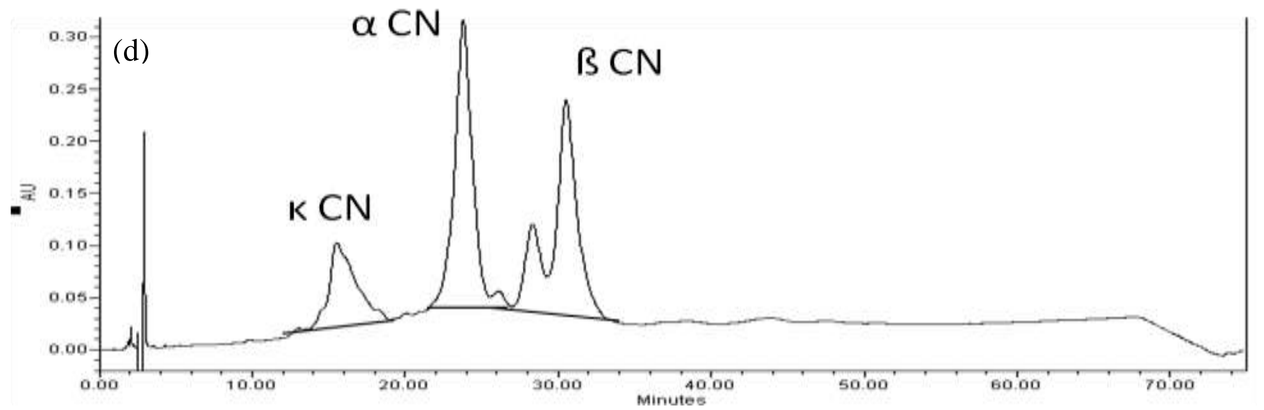
RP-HPLC profile of casein standards, milk proteins and milk protein-Vit A complexes are shown in figure 4.28. α-, ß- and κ-CN were eluted at 23.75, 30.63 and 17.44 min, respectively (Fig 4.28 (a) (b), (c)). All the three casein standards i.e. α-, ß- and κ-CN were combined at a concentration 4.48, 3.48 and 1.22 mg/ml and the RP-HPLC profile is shown in figure 4.28 (d).

Figure 4.28 (e-h) depict the comparison between the RP-HPLC profiles of milk proteins to milk protein-Vit A complexes. The chromatogram of casein standards revealed separate peaks for all the casein fractions (Fig 4.28 (a-d)). RP-HPLC chromatogram of NaCas, RNaCas, NaCas-VA and RNaCas-VA showed sharp peaks for α-, ß- and κ-CN (Fig 4.28 (e-f)). Succinylated protein and succinylated protein-Vit A complexes showed higher retention and slow elution may be due to increased hydrophobicity of succinylated proteins (Fig 4.28 (g-h)).


(a)
α CN
AU
Minutes
0.00
0.05
0.10
0.15
0.20
0.25
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00


(b)
ß CN


(c)
κ CN
AU
Minutes
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00


(d)
α CN
ß CN
κ CN
AU
Minutes
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.00
10.00
20.00
30.00
40.00
50.00
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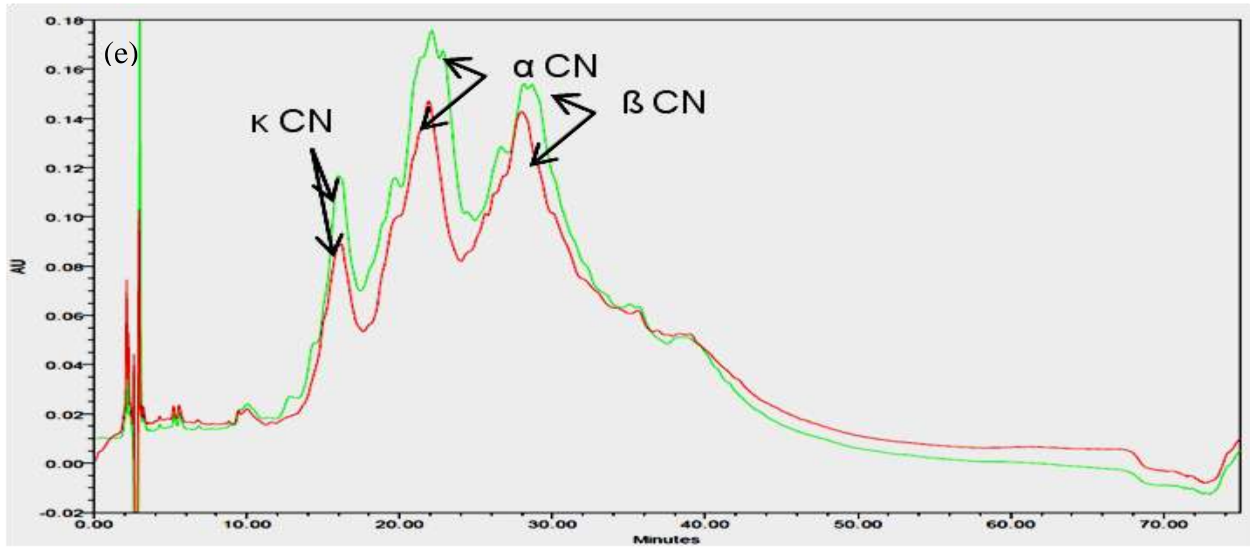
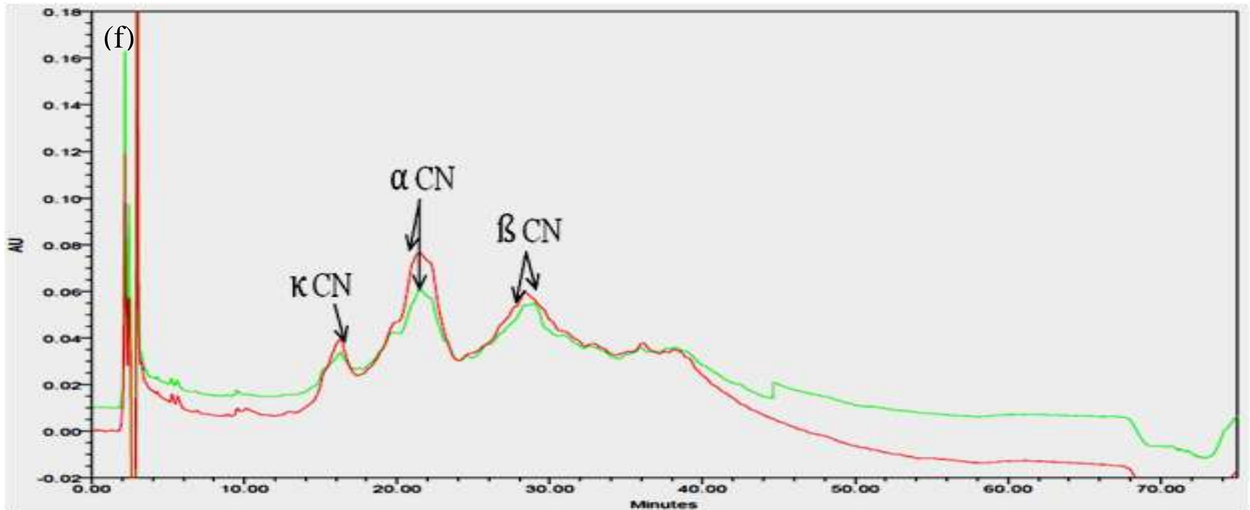
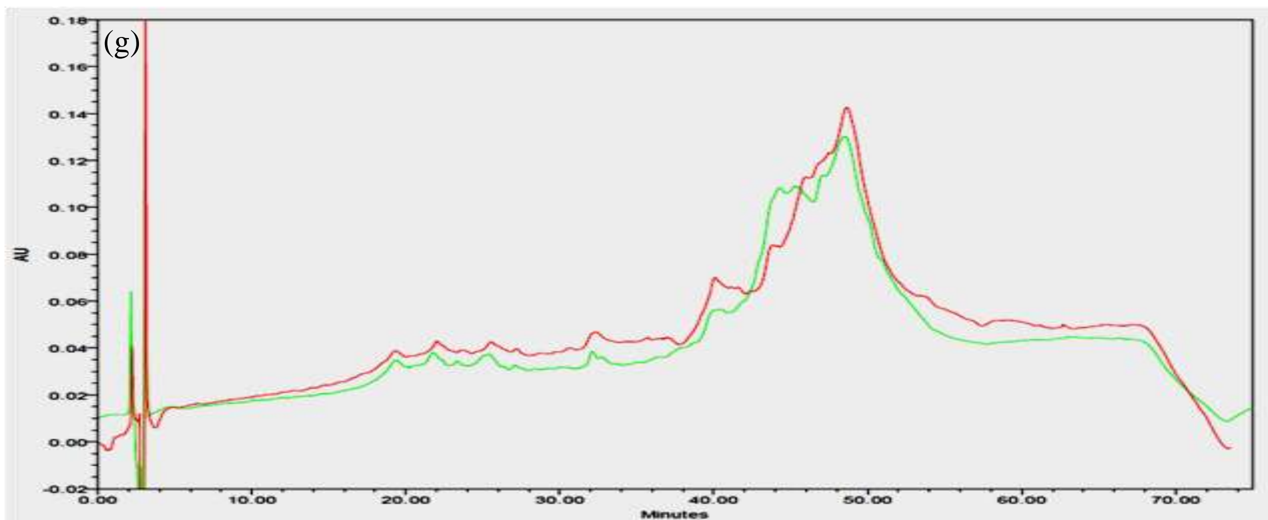
70.00


(e)
κ CN
α CN
ß CN
AU
Minutes


(f)
κ CN
α CN
ß CN
AU
Minutes


(g)
AU
Minutes

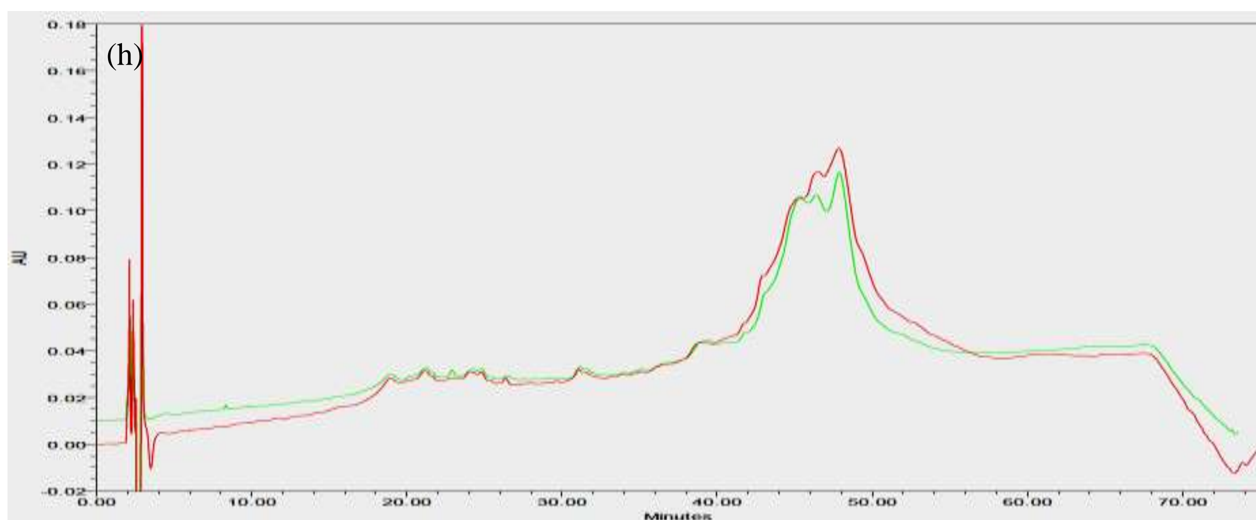



**Fig 4.28 : RP-HPLC profile of milk protein and milk protein-Vit A complexes, (a) α-CN, (b) ß-CN, (c) κ-CN, (d) casein standard, (e) NaCas and NaCas-VA ST complexes (lower to upper), (f) RNaCas and RNaCas-VA complexes (lower to upper) (g) SNaCas and SNaCas-VA ST complexes (lower to upper) and (h) RSNaCas and RSNaCas-VA complexes (lower to upper)**

Sharp peaks for α-, ß- and κ-CN were not observed in these samples, due to merging of peaks as affected by higher retention and slow elution. Binding of vitamin A to milk protein did not have any influence on the RP-HPLC elution profile of different milk proteins. Lakkis and Villota (1992) reported that succinylation increases the hydrophobicity of milk proteins. Meek (1980) reported that more hydrophobic compounds retain more strongly with the stationary phase as compared to less hydrophobic compounds and thus require larger amount of mobile phase for their elution from the column. Our results were in accordance with Jahaniaval *et al.* (2000), Yuksel and Erdem (2010) who reported similar RP-HPLC elution profile of sodium caseinate and bovine casein, respectively. No literature is available regarding the chromatographic behaviour of succinylated protein, reassembled protein and milk protein-Vit A complexes.

### 4.9.2 Evaluation of vitamin A stability in milk protein-Vit A complexes

The stability of vitamin A in milk protein-Vit A complexes was evaluated in comparison to the free vitamin A (oily form). Selected complexes (NaCas-VA ST,

SNaCas-VA ST, RNaCas-VA and RSNaCas-VA) and free vitamin A (oily form) were stored in microcentrifuge tubes (1.5 ml, virgin polypropylene) and aluminium laminate pouches (Polyethylene terephthalate or nylon/Aluminium foil/polypropylene, thickness 0.11 mm). Samples were then evaluated for vitamin A content at a time interval of one month upto six months of storage.

#### 4.9.2.1 Evaluation of vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in aluminium laminate pouches

Milk protein-Vit A complexes and free vitamin A (oily form) were stored in aluminium laminate pouches at three different temperatures i.e. -20, 4 and 37°C, respectively.

##### 4.9.2.1.1 Evaluation of vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in aluminium laminate pouches at -20°C

Vitamin A content in milk protein-vitamin A complexes and free vitamin A (oily form) stored in aluminium laminate pouches at -20°C is presented in Table 4.23 which indicated that vitamin A content significantly reduced ($p<0.05$) during the storage period of six months for all the samples.

**Table 4.23: Vitamin A content in milk protein-Vit A complexes and free vitamin A (oily form) stored in aluminium laminate pouches at -20°C**

| Storage period (months) | Vitamin A content (IU/10 mg) | | | | |
|---|---|---|---|---|---|
| | Milk protein-Vit A complexes | | | | Free vitamin A (oily form) |
| | NaCas-VA ST | SNaCas-VA ST | RNaCas-VA | RSNaCas-VA | |
| **0** | 1040.01±26.28$^{f}$ | 845.52±30.05$^{e}$ | 682.89±16.79$^{e}$ | 645.01±26.28$^{d}$ | 16366.37±198.34$^{g}$ |
| **1** | 958.75±8.10$^{e}$ | 802.72±7.94$^{d}$ | 671.56±5.25$^{e}$ | 641.32±2.28$^{d}$ | 14649.73±210.11$^{f}$ |
| **2** | 909.49±9.23$^{d}$ | 763.33±4.17$^{c}$ | 639.08±5.21$^{d}$ | 606.82±8.35$^{c}$ | 13465.09±159.66$^{e}$ |
| **3** | 870.55±5.77$^{c}$ | 721.52±4.60$^{b}$ | 599.40±4.69$^{c}$ | 574.62±4.85$^{c}$ | 12756.35±182.41$^{d}$ |
| **4** | 822.63±1.55$^{b}$ | 688.43±5.33$^{b}$ | 570.64±8.92$^{b}$ | 546.08±3.13$^{b}$ | 11788.06±55.51$^{c}$ |
| **5** | 768.01±9.69$^{a}$ | 649.65±5.88$^{a}$ | 532.82±7.29$^{a}$ | 517.61±2.92$^{ab}$ | 10666.41±136.68$^{b}$ |
| **6** | 734.68±8.14$^{a}$ | 630.67±7.53$^{a}$ | 524.21±8.17$^{a}$ | 502.10±2.59$^{a}$ | 10109.29±97.79$^{a}$ |

Data are presented as means±SEM (n=3)
$^{a-b}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.

Vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in aluminium laminate pouches at -20°C is presented in Table 4.24, Fig 4.29 and

4.30. Free vitamin A (oily form), NaCas-VA ST, SNaCas-VA ST, RNaCas-VA and RSNaCas-VA showed 89.51, 92.19, 94.98, 98.34 and 99.43% vitamin A stability after one month of storage and 61.77, 70.64, 74.59, 76.76 and 77.84% vitamin A stability after six months of storage. Vitamin A stability in all milk protein-Vit A complexes and free vitamin A (oily form) was significantly different ($p<0.05$) from each other during the storage period of six months. Free vitamin A (oily form) showed lowest stability followed by NaCas-VA ST, SNaCas-VA ST, RNaCas-VA and RSNaCas-VA complexes. Binding of free vitamin A (oily form) to milk protein improved the vitamin A stability. Reassembled milk protein-Vit A complexes showed higher vitamin A stability as compared to the complexes prepared by stirring method. All milk protein-Vit A complexes and free vitamin A (oily form) showed significant decrease ($p<0.05$) in vitamin A stability during the storage period of six months.

**Table 4.24: Vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in aluminium laminate pouches at -20°C**

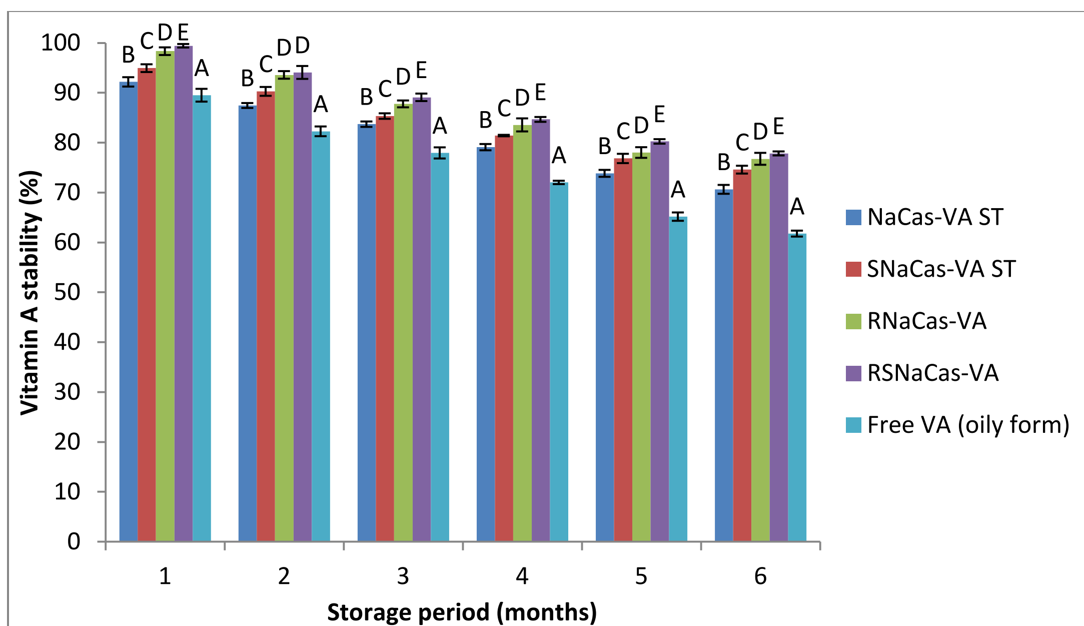
| Storage period (months) | Vitamin A stability (%) | | | | |
|---|---|---|---|---|---|
| | Milk protein-Vit A complexes | | | | Free vitamin A (oily form) |
| | NaCas-VA ST | SNaCas-VA ST | RNaCas-VA | RSNaCas-VA | |
| **1** | 92.19±0.78$^{fB}$ | 94.98±0.94$^{fC}$ | 98.34±0.77$^{fD}$ | 99.43±0.35$^{fE}$ | 89.51±1.28$^{fA}$ |
| **2** | 87.45±0.89$^{eB}$ | 90.28±0.49$^{eC}$ | 93.58±0.76$^{eD}$ | 94.08±1.29$^{eD}$ | 82.27±0.98$^{eA}$ |
| **3** | 83.71±0.55$^{dB}$ | 85.33±0.54$^{dC}$ | 87.77±0.69$^{dD}$ | 89.09±0.75$^{dE}$ | 77.94±1.11$^{dA}$ |
| **4** | 79.10±0.15$^{cB}$ | 81.42±0.63$^{cC}$ | 83.56±1.31$^{cD}$ | 84.66±0.49$^{cE}$ | 72.03±0.34$^{Ca}$ |
| **5** | 73.85±0.93$^{bB}$ | 76.83±0.70$^{bC}$ | 78.02±1.07$^{bD}$ | 80.25±0.45$^{bE}$ | 65.17±0.84$^{Ba}$ |
| **6** | 70.64±0.78$^{aB}$ | 74.59±0.89$^{aC}$ | 76.76±1.20$^{aD}$ | 77.84±0.40$^{aE}$ | 61.77±0.60$^{aA}$ |

Data are presented as means±SEM (n=3)
$^{a-b}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.
$^{A-B}$Means within rows with different superscript are significantly different ($p<0.05$) from each other.

#### 4.9.2.1.2 Evaluation of vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in aluminium laminate pouches at 4°C

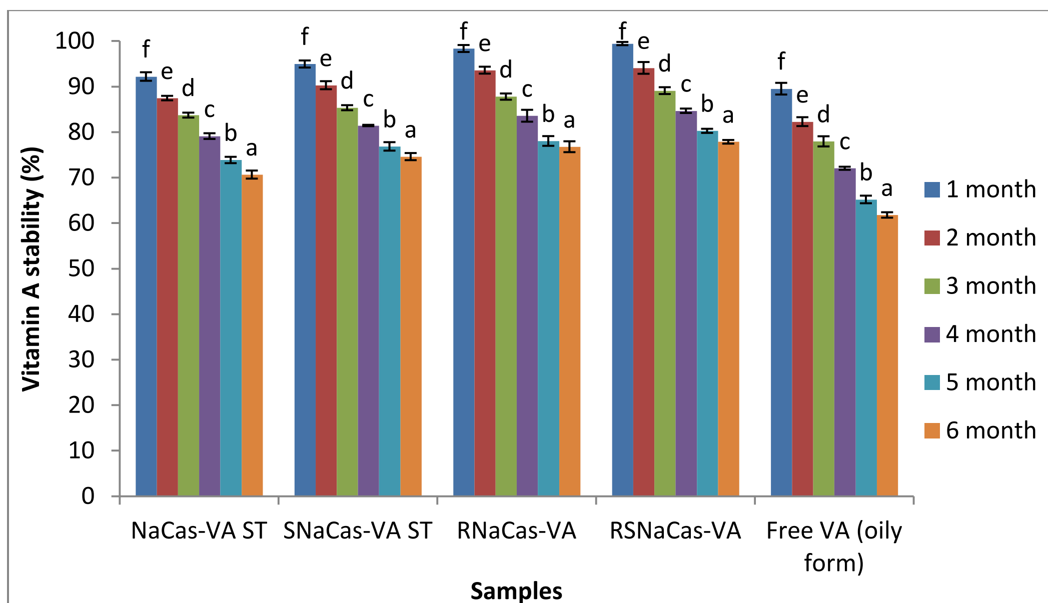
Vitamin A content in milk protein-vitamin A complexes and free vitamin A (oily form) stored in aluminium laminate pouches at 4°C is presented in Table 4.25 which indicated that vitamin A content significantly reduced ($p<0.05$) during the storage period of six months for all the samples.

Vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in aluminium laminate pouches at 4°C is presented in Table 4.26, figure 4.31 and




[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.

**Fig 4.29: Comparison of vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in aluminium laminate pouches at -20°C**




[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.

**Fig 4.30: Vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) as affected by storage in aluminium laminate pouches at -20°C**

**Table 4.25: Vitamin A content in milk protein-Vit A complexes and free vitamin A (oily form) stored in aluminium laminate pouches at 4°C**

| Storage period (months) | Vitamin A content (IU/10 mg) | | | | |
|---|---|---|---|---|---|
| | Milk protein-Vit A complexes | | | | Free vitamin A (oily form) |
| | NaCas-VA ST | SNaCas-VA ST | RNaCas-VA | RSNaCas-VA | |
| 0 | 1040.01±26.28$^{f}$ | 845.52±30.05$^{d}$ | 682.89±16.79$^{f}$ | 645.01±26.28$^{e}$ | 16366.37±198.34$^{g}$ |
| 1 | 882.29±3.11$^{e}$ | 736.23±8.76$^{c}$ | 610.43±4.24$^{e}$ | 582.14±5.99$^{d}$ | 13321.20±129.45$^{f}$ |
| 2 | 832.74±9.24$^{d}$ | 710.81±6.71$^{c}$ | 582.81±3.67$^{d}$ | 558.63±4.25$^{cd}$ | 12325.24±149.39$^{e}$ |
| 3 | 796.48±3.84$^{c}$ | 653.76±3.23$^{b}$ | 547.82±7.29$^{c}$ | 526.23±3.05$^{bc}$ | 11628.01±234.07$^{d}$ |
| 4 | 754.69±4.36$^{b}$ | 640.16±5.13$^{b}$ | 528.64±8.07$^{bc}$ | 506.96±8.66$^{b}$ | 11149.67±150.51$^{c}$ |
| 5 | 734.02±3.67$^{ab}$ | 600.48±8.11$^{a}$ | 516.53±9.11$^{ab}$ | 494.62±1.36$^{ab}$ | 9970.98±100.01$^{b}$ |
| 6 | 716.36±4.60$^{a}$ | 586.42±4.32$^{a}$ | 496.06±2.06$^{a}$ | 471.38±3.45$^{a}$ | 9076.98±125.29$^{a}$ |

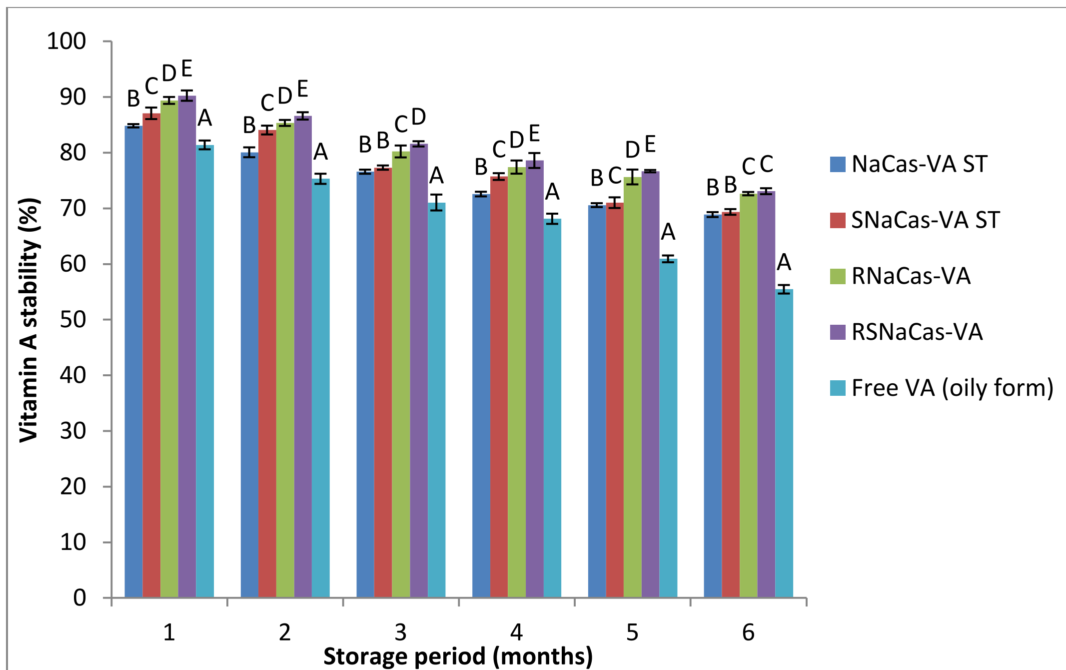
Data are presented as means±SEM (n=3)
$^{a-b}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.

4.32. Free vitamin A (oily form), NaCas-VA ST, SNaCas-VA ST, RNaCas-VA and RSNaCas-VA showed 81.39, 84.84, 87.07, 89.39 and 90.25% vitamin A stability after one month of storage and 55.46, 68.88, 69.36, 72.64 and 73.08% vitamin A stability after six months of storage. Vitamin A stability in all milk protein-Vit A complexes and free vitamin A (oily form) was significantly different ($p<0.05$) from each other during the storage period of six months. At 4°C, vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) followed similar trend as reported at -20°C. All milk protein-Vit A complexes and free vitamin A (oily form) showed significant decrease ($p<0.05$) in vitamin A stability during the storage period of six months.

**Table 4.26: Vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in aluminium laminate pouches at 4°C**

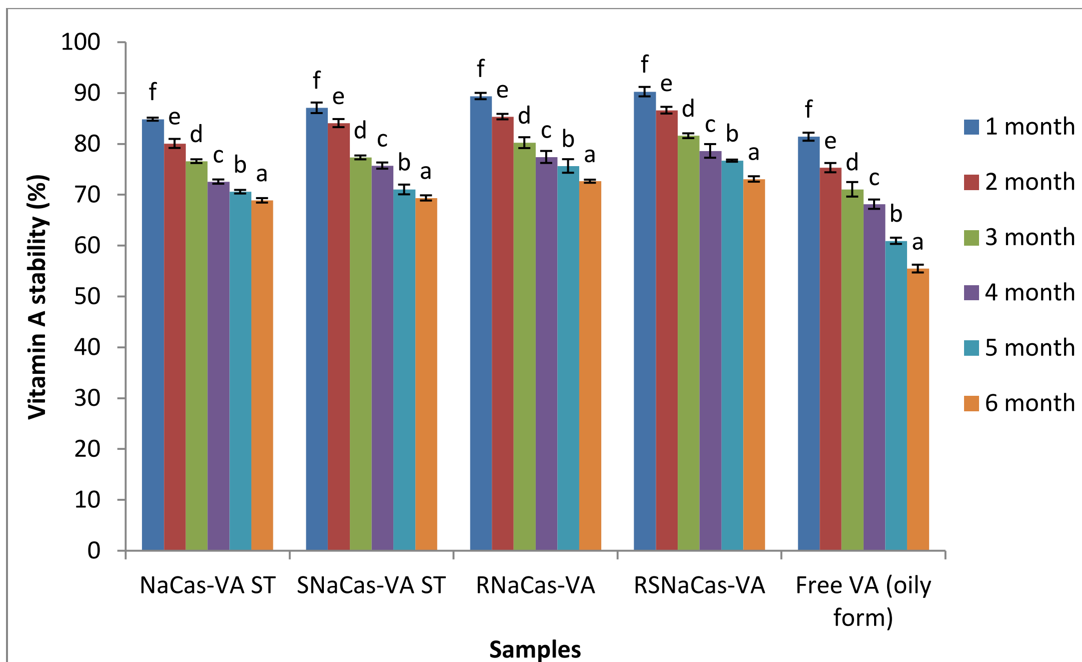
| Storage period (months) | Vitamin A stability (%) | | | | |
|---|---|---|---|---|---|
| | Milk protein-Vit A complexes | | | | Free vitamin A (oily form) |
| | NaCas-VA ST | SNaCas-VA ST | RNaCas-VA | RSNaCas-VA | |
| 1 | 84.84±0.30$^{fB}$ | 87.07±1.04$^{fC}$ | 89.39±0.62$^{fD}$ | 90.25±0.93$^{fE}$ | 81.39±0.79$^{Fa}$ |
| 2 | 80.07±0.89$^{eB}$ | 84.07±0.79$^{eC}$ | 85.34±0.53$^{eD}$ | 86.61±0.66$^{eE}$ | 75.31±0.91$^{eA}$ |
| 3 | 76.58±0.37$^{dB}$ | 77.32±0.38$^{dB}$ | 80.22±1.06$^{dC}$ | 81.58±1.07$^{dD}$ | 71.05±1.43$^{dA}$ |
| 4 | 72.57±0.42$^{cB}$ | 75.71±0.61$^{cC}$ | 77.41±1.18$^{cD}$ | 78.59±1.18$^{cE}$ | 68.13±0.92$^{cA}$ |
| 5 | 70.58±0.35$^{bB}$ | 71.02±0.96$^{bC}$ | 75.64±1.33$^{bD}$ | 76.68±1.33$^{bE}$ | 60.92±0.61$^{bA}$ |
| 6 | 68.88±0.44$^{aB}$ | 69.36±0.51$^{aB}$ | 72.64±0.30$^{aC}$ | 73.08±0.30$^{aC}$ | 55.46±0.77$^{aA}$ |

Data are presented as means±SEM (n=3)
$^{a-b}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.
$^{A-B}$Means within rows with different superscript are significantly different ($p<0.05$) from each other.




[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.

**Fig 4.31: Comparison of vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in aluminium laminate pouches at 4°C**




[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.

**Fig 4.32: Vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) as affected by storage in aluminium laminate pouches at 4°C**

### 4.9.2.1.3 Evaluation of vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in aluminium laminate pouches at 37°C

Vitamin A content in milk protein-vitamin A complexes and free vitamin A (oily form) stored in aluminium laminate pouches at 37°C is presented in Table 4.27 which indicated that vitamin A content significantly reduced ($p<0.05$) during the storage period of six months for all the samples.

**Table 4.27: Vitamin A content in milk protein-Vit A complexes and free vitamin A (oily form) stored in aluminium laminate pouches at 37°C**

| Storage period (months) | Vitamin A content (IU/10 mg) | | | | |
|---|---|---|---|---|---|
| | Milk protein-Vit A complexes | | | | Free vitamin A (oily form) |
| | NaCas-VA ST | SNaCas-VA ST | RNaCas-VA | RSNaCas-VA | |
| 0 | 1040.01±26.28$^{g}$ | 845.52±30.05$^{f}$ | 682.89±16.79$^{g}$ | 645.01±26.28$^{f}$ | 16366.37±198.34$^{g}$ |
| 1 | 588.43±9.08$^{f}$ | 502.76±6.79$^{e}$ | 418.25±3.96$^{f}$ | 407.83±2.08$^{e}$ | 8304.98±125.06$^{f}$ |
| 2 | 291.92±3.45$^{e}$ | 263.41±1.91$^{d}$ | 241.87±3.45$^{e}$ | 248.04±3.52$^{d}$ | 4068.13±148.01$^{e}$ |
| 3 | 224.81±4.70$^{d}$ | 192.00±2.50$^{c}$ | 176.53±2.01$^{d}$ | 175.46±2.05$^{c}$ | 2888.81±69.60$^{d}$ |
| 4 | 164.48±5.80$^{c}$ | 134.99±2.85$^{b}$ | 120.71±2.31$^{c}$ | 120.10±2.26$^{b}$ | 1630.42±91.81$^{c}$ |
| 5 | 100.58±5.81$^{b}$ | 83.97±3.66$^{a}$ | 72.12±2.90$^{b}$ | 69.10±6.17$^{a}$ | 777.61±37.07$^{b}$ |
| 6 | 60.61±3.88$^{a}$ | 50.75±2.79$^{a}$ | 46.07±2.01$^{a}$ | 45.73±3.28$^{a}$ | 51.14±9.27$^{a}$ |

Data are presented as means±SEM (n=3)

$^{a-b}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.

Vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in aluminium laminate pouches at 37°C is presented in Table 4.28, Fig 4.33 and

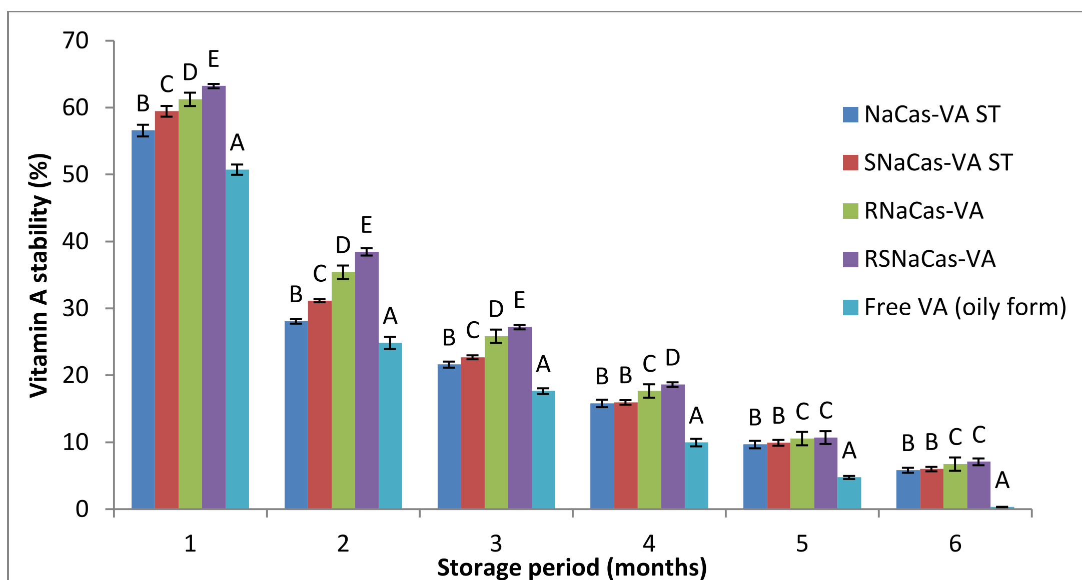
**Table 4.28: Vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in aluminium laminate pouches at 37°C**

| Storage period (months) | Vitamin A stability (%) | | | | |
|---|---|---|---|---|---|
| | Milk protein-Vit A complexes | | | | Free vitamin A (oily form) |
| | NaCas-VA ST | SNaCas-VA ST | RNaCas-VA | RSNaCas-VA | |
| 1 | 56.58±0.87$^{fB}$ | 59.46±0.80$^{fC}$ | 61.25±0.58$^{fD}$ | 63.23±0.32$^{fE}$ | 50.74±0.76$^{fA}$ |
| 2 | 28.07±0.33$^{eB}$ | 31.15±0.23$^{eC}$ | 35.42±0.51$^{eD}$ | 38.46±0.55$^{eE}$ | 24.86±0.90$^{eA}$ |
| 3 | 21.62±0.45$^{dB}$ | 22.70±0.30$^{dC}$ | 25.85±0.29$^{dD}$ | 27.20±0.32$^{dE}$ | 17.65±0.43$^{dA}$ |
| 4 | 15.82±0.56$^{cB}$ | 15.97±0.34$^{cB}$ | 17.68±0.34$^{cC}$ | 18.62±0.35$^{cD}$ | 9.96±0.56$^{cA}$ |
| 5 | 9.67±0.56$^{bB}$ | 9.93±0.43$^{bB}$ | 10.56±0.43$^{bC}$ | 10.71±0.96$^{bC}$ | 4.75±0.23$^{bA}$ |
| 6 | 5.83±0.37$^{aB}$ | 6.00±0.33$^{aB}$ | 6.75±0.29$^{aC}$ | 7.09±0.51$^{aC}$ | 0.31±0.06$^{aA}$ |

Data are presented as means±SEM (n=3)

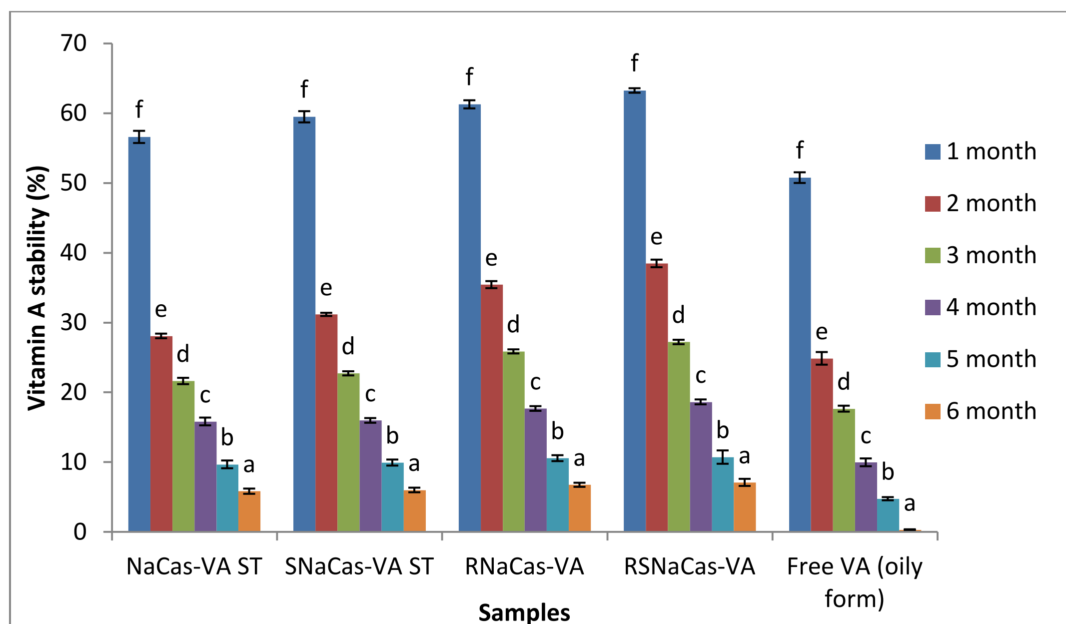
$^{a-b}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.

$^{A-B}$Means within rows with different superscript are significantly different ($p<0.05$) from each other.




[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.

**Fig 4.33: Comparison of vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in aluminium laminate pouches at 37°C**




[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.

**Fig 4.34: Vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) as affected by storage in aluminium laminate pouches at 37°C**

4.34. Free vitamin A (oily form), NaCas-VA ST, SNaCas-VA ST, RNaCas-VA and RSNaCas-VA showed 50.74, 56.58, 59.46, 61.25 and 63.23% vitamin A stability after one month of storage and 0.31, 5.83, 6.00, 6.75 and 7.09% vitamin A stability after six months of storage. Vitamin A stability in all milk protein-Vit A complexes and free vitamin A (oily form) was significantly different ($p<0.05$) from each other during the storage period of six months. At 37°C, vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) followed similar trend as reported at -20 and 4°C. All milk protein-Vit A complexes and free vitamin A (oily form) showed significant decrease ($p<0.05$) in vitamin A stability during the storage period of six months.

Milk protein-Vit A complexes and free vitamin A (oily form) showed lowest vitamin A stability at 37°C followed by 4°C and -20°C (Table 4.24, 4.26, 4.28). Our results were in accordance with Chavez-Servin *et al.* (2008a) who reported that infant milk based powdered formula fortified with vitamin A, E and C, iron and selenium showed higher vitamin A degradation at higher temperature. Maguer and Jackson (1983) also reported that vitamin A fortified UHT processed milk showed higher vitamin A stability at 20°C as compared to 35°C.

#### 4.9.2.2 Evaluation of vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in microcentrifuge tubes (polypropylene)

Milk protein-Vit A complexes and free vitamin A (oily form) were stored in microcentrifuge tubes at three different temperatures i.e -20, 4 and 37°C.

##### 4.9.2.2.1 Evaluation of vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in microcentrifuge tubes (polypropylene) at -20°C

Vitamin A content in milk protein-vitamin A complexes and free vitamin A (oily form) stored in microcentrifuge tubes at -20°C is presented in Table 4.29 which indicated that vitamin A content significantly reduced ($p<0.05$) during the storage period of six months for all the samples.

Vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in microcentrifuge tubes at -20°C is presented in Table 4.30, Fig 4.35 and 4.36. Free vitamin A (oily form), NaCas-VA ST, SNaCas-VA ST, RNaCas-VA and RSNaCas-VA showed 77.65, 79.21, 81.82, 84.78 and 86.97% vitamin A stability after one month

**Table 4.29: Vitamin A content in milk protein-Vit A complexes and free vitamin A (oily form) stored in microcentrifuge tubes (polypropylene) at -20°C**

| Storage period (months) | Vitamin A content (IU/10 mg) | | | | |
|---|---|---|---|---|---|
| | Milk protein-Vit A complexes | | | | Free vitamin A (oily form) |
| | NaCas-VA ST | SNaCas-VA ST | RNaCas-VA | RSNaCas-VA | |
| 0 | 1040.01±26.28$^{f}$ | 845.52±30.05$^{d}$ | 682.89±16.79$^{e}$ | 645.01±26.28$^{d}$ | 16366.67±198.34$^{f}$ |
| 1 | 823.77±5.05$^{e}$ | 691.84±3.85$^{c}$ | 578.94±6.96$^{d}$ | 560.98±3.21$^{c}$ | 12708.50±194.43$^{e}$ |
| 2 | 781.21±7.92$^{d}$ | 645.66±10.49$^{c}$ | 537.99±7.91$^{c}$ | 533.95±3.93$^{c}$ | 11834.81±110.25$^{d}$ |
| 3 | 702.40±6.62$^{c}$ | 600.15±12.77$^{b}$ | 515.03±5.53$^{bc}$ | 516.56±18.06$^{b}$ | 10631.21±288.99$^{c}$ |
| 4 | 683.31±9.29$^{bc}$ | 567.24±15.15$^{b}$ | 483.52±8.01$^{ab}$ | 515.61±11.13$^{b}$ | 9997.83±72.56$^{b}$ |
| 5 | 663.93±5.56$^{ab}$ | 556.65±14.06$^{a}$ | 470.54±30.37$^{a}$ | 493.78±5.32$^{ab}$ | 9701.54±124.10$^{ab}$ |
| 6 | 642.48±2.86$^{a}$ | 526.39±5.61$^{a}$ | 451.37±16.35$^{a}$ | 471.48±6.49$^{a}$ | 9302.44±25.05$^{a}$ |

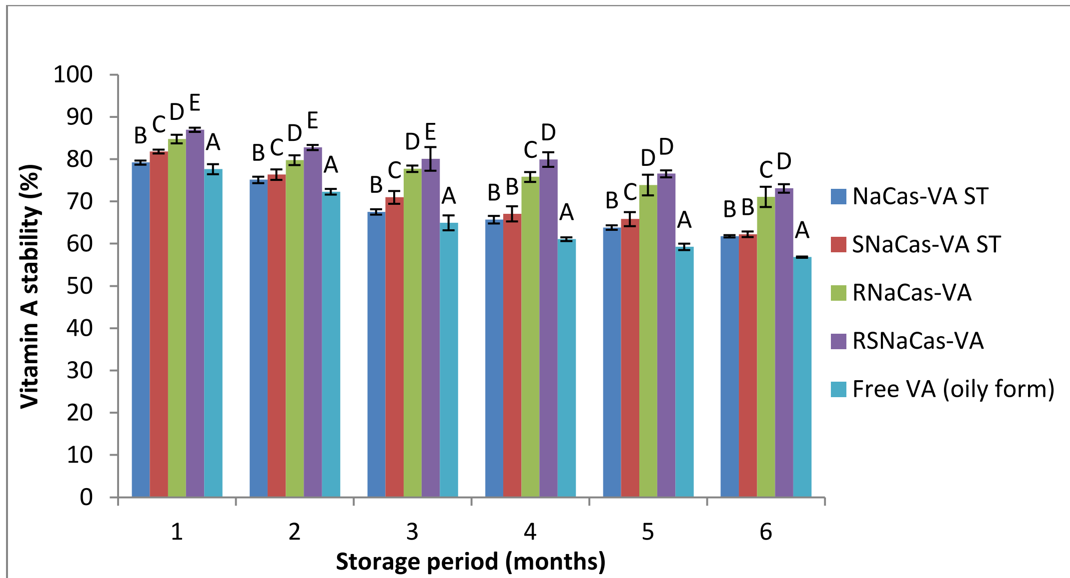
Data are presented as means±SEM (n=3)
$^{a-b}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.

of storage and 56.84, 61.78, 62.26, 71.09 and 73.10% vitamin A stability after six months of storage. Vitamin A stability in all milk protein-Vit A complexes and free vitamin A (oily form) was significantly different ($p<0.05$) from each other during the storage period of six months. Free vitamin A (oily form) showed lowest stability followed by NaCas-VA ST, SNaCas-VA ST, RNaCas-VA and RSNaCas-VA complexes. Binding of free vitamin A (oily form) to milk protein improved the vitamin A stability. Reassembled milk protein-Vit A complexes showed higher vitamin A stability as compared to the complexes prepared by stirring method. All milk protein-Vit A complexes and free vitamin A (oily form), showed significant decrease ($p<0.05$) in vitamin A stability during the storage period of six months.

**Table 4.30: Vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in microcentrifuge tubes (polypropylene) at -20°C**

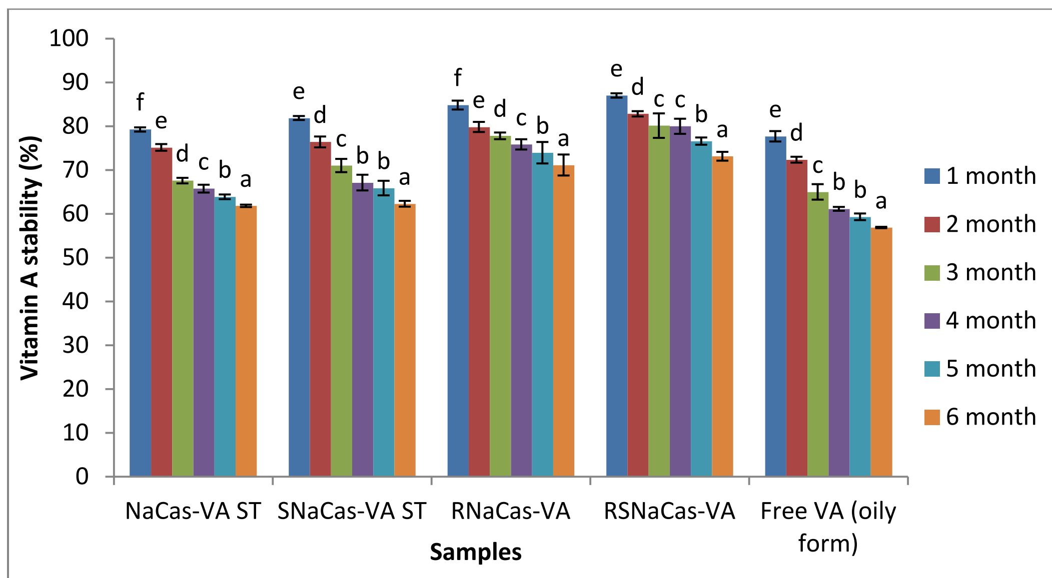
| Storage period (months) | Vitamin A stability (%) | | | | |
|---|---|---|---|---|---|
| | Milk protein-Vit A complexes | | | | Free vitamin A (oily form) |
| | NaCas-VA ST | SNaCas-VA ST | RNaCas-VA | RSNaCas-VA | |
| 1 | 79.21±0.49$^{fB}$ | 81.82±0.45$^{eC}$ | 84.78±1.02$^{fD}$ | 86.97±0.50$^{eE}$ | 77.65±1.19$^{eA}$ |
| 2 | 75.12±0.76$^{eB}$ | 76.36±1.24$^{dC}$ | 79.78±1.16$^{eD}$ | 82.78±0.61$^{dE}$ | 72.31±0.67$^{dA}$ |
| 3 | 67.54±0.64$^{dB}$ | 70.98±1.51$^{cC}$ | 77.75±0.76$^{dD}$ | 80.08±2.80$^{cE}$ | 64.96±1.77$^{cA}$ |
| 4 | 65.70±0.89$^{cB}$ | 67.09±1.79$^{bB}$ | 75.80±1.17$^{cC}$ | 79.93±1.73$^{cD}$ | 61.08±0.44$^{bA}$ |
| 5 | 63.84±0.53$^{bB}$ | 65.83±1.66$^{bC}$ | 73.90±2.44$^{bD}$ | 76.55±0.83$^{bD}$ | 59.28±0.76$^{bA}$ |
| 6 | 61.78±0.27$^{aB}$ | 62.26±0.66$^{aB}$ | 71.09±2.39$^{aC}$ | 73.10±1.00$^{aD}$ | 56.84±0.15$^{aA}$ |

Data are presented as means±SEM (n=3)
$^{a-b}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.
$^{A-B}$Means within rows with different superscript are significantly different ($p<0.05$) from each other.




[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.

**Fig 4.35: Comparison of vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in microcentrifuge tubes (polypropylene) at -20°C**




[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.

**Fig 4.36: Vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) as affected by storage in microcentrifuge tubes (polypropylene) at -20°C**

**4.9.2.2.2 Evaluation of vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in microcentrifuge tubes (polypropylene) at 4°C**

Vitamin A content in milk protein-vitamin A complexes and free vitamin A (oily form) stored in microcentrifuge tubes at 4°C is presented in Table 4.31 which indicated that vitamin A content significantly reduced ($p<0.05$) during the storage period of six months for all the samples.

**Table 4.31: Vitamin A content in milk protein-Vit A complexes and free vitamin A (oily form) stored in microcentrifuge tubes (polypropylene) at 4°C**

| Storage period (months) | Vitamin A content (IU/10 mg) | | | | |
|---|---|---|---|---|---|
| | Milk protein-Vit A complexes | | | | Free vitamin A (oily form) |
| | NaCas-VA ST | SNaCas-VA ST | RNaCas-VA | RSNaCas-VA | |
| **0** | 1040.01±26.28$^{f}$ | 845.52±30.05$^{f}$ | 682.89±16.79$^{d}$ | 645.01±26.28$^{f}$ | 16366.37±198.34$^{f}$ |
| **1** | 756.90±3.40$^{e}$ | 632.09±5.27$^{e}$ | 531.81±13.64$^{c}$ | 520.91±5.11$^{e}$ | 11433.82±155.86$^{e}$ |
| **2** | 605.21±3.50$^{d}$ | 506.57±7.92$^{d}$ | 423.10±1.92$^{b}$ | 423.19±5.01$^{d}$ | 9065.23±206.87$^{d}$ |
| **3** | 558.64±9.24$^{c}$ | 469.59±3.05$^{c}$ | 404.78±5.56$^{b}$ | 410.94±1.65$^{cd}$ | 8225.83±187.46$^{c}$ |
| **4** | 521.68±4.91$^{b}$ | 434.06±8.30$^{b}$ | 371.54±2.45$^{a}$ | 391.73±7.23$^{bc}$ | 7658.18±84.23$^{b}$ |
| **5** | 508.59±9.73$^{b}$ | 414.47±7.93$^{ab}$ | 360.28±5.26$^{a}$ | 371.91±5.27$^{ab}$ | 7361.35±48.59$^{b}$ |
| **6** | 465.81±4.14$^{a}$ | 397.34±3.36$^{a}$ | 349.83±5.09$^{a}$ | 354.91±3.87$^{a}$ | 6747.86±121.59$^{a}$ |

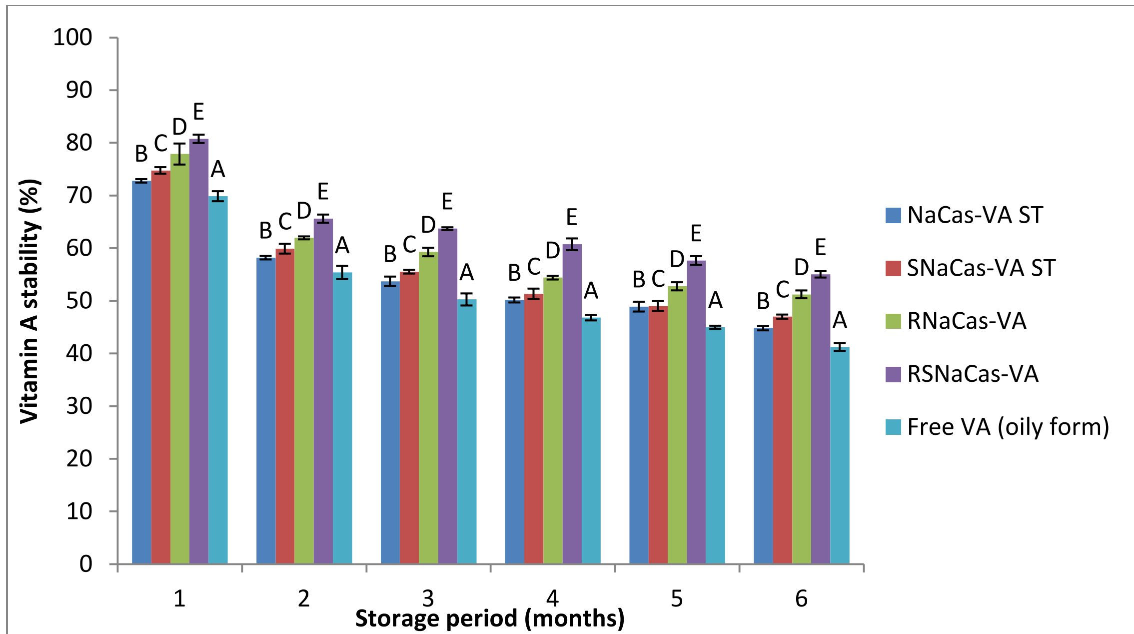
Data are presented as means±SEM (n=3)
$^{a-b}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.

Vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in microcentrifuge tubes at 4°C is presented in Table 4.32, Fig 4.37 and 4.38.

**Table 4.32: Vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in microcentrifuge tubes (polypropylene) at 4°C**

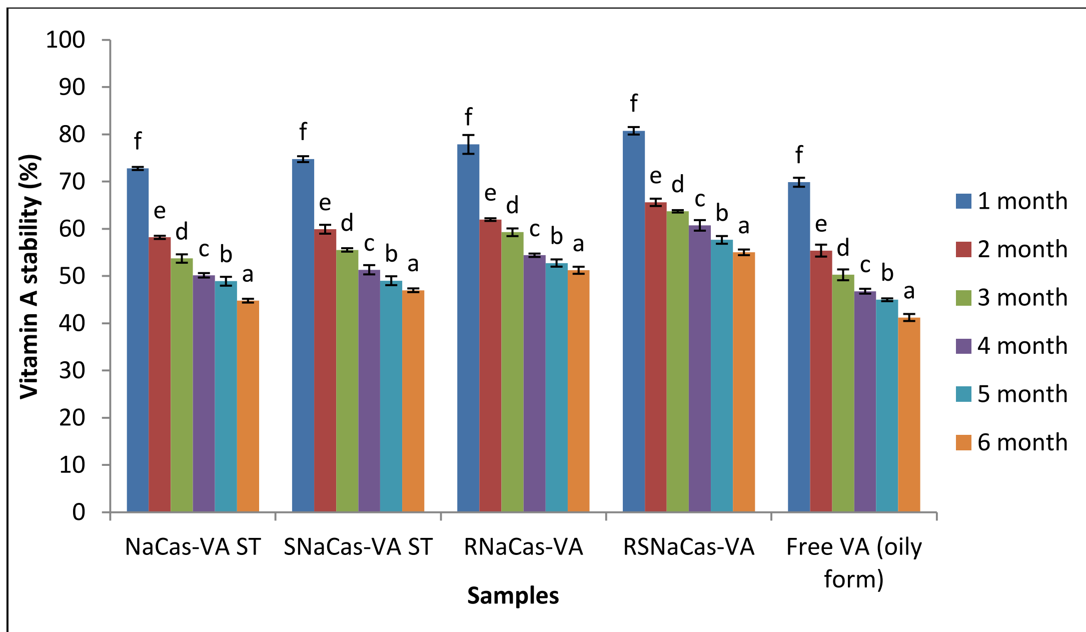
| Storage period (months) | Vitamin A stability (%) | | | | |
|---|---|---|---|---|---|
| | Milk protein-Vit A complexes | | | | Free vitamin A (oily form) |
| | NaCas-VA ST | SNaCas-VA ST | RNaCas-VA | RSNaCas-VA | |
| **1** | 72.78±0.33$^{fB}$ | 74.76±0.62$^{fC}$ | 77.88±1.99$^{fD}$ | 80.76±0.79$^{fE}$ | 69.86±0.95$^{fA}$ |
| **2** | 58.19±0.34$^{eB}$ | 59.91±0.94$^{eC}$ | 61.96±0.28$^{eD}$ | 65.61±0.78$^{eE}$ | 55.39±1.26$^{eA}$ |
| **3** | 53.72±0.89$^{dB}$ | 55.54±0.36$^{dC}$ | 59.27±0.81$^{dD}$ | 63.71±0.26$^{dE}$ | 50.26±1.15$^{dA}$ |
| **4** | 50.16±0.47$^{cB}$ | 51.34±0.98$^{cC}$ | 54.41±0.36$^{cD}$ | 60.73±1.12$^{cE}$ | 46.79±0.51$^{cA}$ |
| **5** | 48.90±0.94$^{bB}$ | 49.02±0.94$^{bC}$ | 52.76±0.77$^{bD}$ | 57.66±0.82$^{bE}$ | 44.98±0.30$^{bA}$ |
| **6** | 44.79±0.40$^{aB}$ | 46.99±0.40$^{aC}$ | 51.23±0.74$^{aD}$ | 55.02±0.60$^{aE}$ | 41.23±0.74$^{aA}$ |

Data are presented as means±SEM (n=3)
$^{a-b}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.
$^{A-B}$Means within rows with different superscript are significantly different ($p<0.05$) from each other.




[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.

**Fig 4.37: Comparison of vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in microcentrifuge tubes (polypropylene) at 4°C**




[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.

**Fig 4.38: Vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) as affected by storage in microcentrifuge tubes (polypropylene) at 4°C**

Free vitamin A (oily form), NaCas-VA ST, SNaCas-VA ST, RNaCas-VA and RSNaCas-VA showed 69.86, 72.78, 74.76, 77.88 and 80.76% vitamin A stability after one month of storage and 41.23, 44.79, 46.99, 51.23 and 55.02% vitamin A stability after six months of storage. Vitamin A stability in all milk protein-Vit A complexes and free vitamin A (oily form) was significantly different ($p<0.05$) from each other during the storage period of six months. At 4°C, vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) followed similar trend as reported at -20°C. All milk protein-Vit A complexes and free vitamin A (oily form) showed significant decrease ($p<0.05$) in vitamin A stability during the storage period of six months.

#### 4.9.2.3.2 Evaluation of vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in microcentrifuge tubes (polypropylene) at 37°C

Vitamin A content in milk protein-vitamin A complexes and free vitamin A (oily form) stored in microcentrifuge tubes at 37°C is presented in Table 4.33 which indicated that vitamin A content significantly reduced ($p<0.05$) during the storage period of six months for all the samples.

**Table 4.33: Vitamin A content in milk protein-Vit A complexes and free vitamin A (oily form) stored in microcentrifuge tubes (polypropylene) at 37°C**

| Storage period (months) | Vitamin A content (IU/10 mg) | | | | |
|---|---|---|---|---|---|
| | Milk protein-Vit A complexes | | | | Free vitamin A (oily form) |
| | NaCas-VA ST | SNaCas-VA ST | RNaCas-VA | RSNaCas-VA | |
| 0 | 1040.01±26.28$^{b}$ | 845.52±30.05$^{b}$ | 645.01±16.79$^{b}$ | 682.89±26.28$^{b}$ | 16366.37±198.34$^{b}$ |
| 1 | 16.90±0.37$^{a}$ | 14.35±0.08$^{a}$ | 16.33±0.19$^{a}$ | 13.03±0.66$^{a}$ | 183.99±13.83$^{a}$ |
| 2 | 13.28±0.55$^{a}$ | 11.28±0.34$^{a}$ | 10.15±0.73$^{a}$ | 10.10±0.87$^{a}$ | 97.91±15.62$^{a}$ |
| 3 | 8.36±0.32$^{a}$ | 8.90±0.51$^{a}$ | 7.22±0.72$^{a}$ | 7.28±0.52$^{a}$ | 28.07±7.18$^{a}$ |
| 4 | 6.46±0.57$^{a}$ | 7.27±0.45$^{a}$ | 6.50±0.63$^{a}$ | 6.61±0.17$^{a}$ | 14.37±1.78$^{a}$ |
| 5 | 4.35±0.15$^{a}$ | 6.23±0.36$^{a}$ | 5.46±0.56$^{a}$ | 5.25±0.21$^{a}$ | 4.58±1.04$^{a}$ |
| 6 | 1.81±0.06$^{a}$ | 2.57±0.29$^{a}$ | 5.08±0.16$^{a}$ | 4.09±0.13$^{a}$ | 3.43±0.78$^{a}$ |

Data are presented as means±SEM (n=3)
$^{a-b}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.

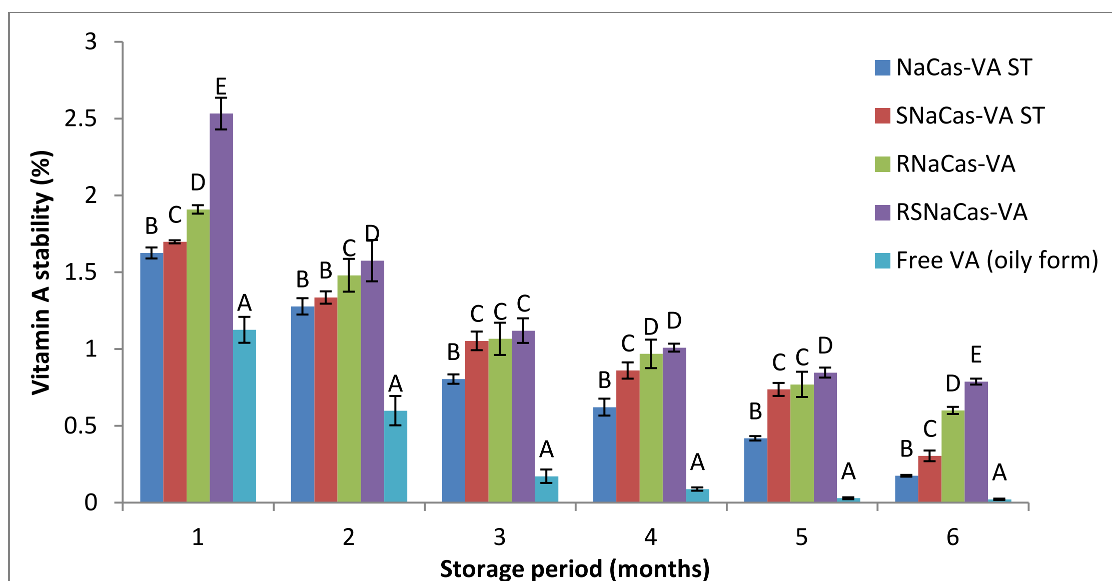
Vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in microcentrifuge tubes at 37°C is presented in Table 4.34, Fig 4.39 and 4.40. Free vitamin A (oily form), NaCas-VA ST, SNaCas-VA ST, RNaCas-VA and RSNaCas-VA showed 1.12, 1.62, 1.70, 1.91 and 2.53% vitamin A stability after one month of

**Table 4.34: Vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in microcentrifuge tubes (polypropylene) at 37°C**

| Storage period (months) | Vitamin A stability (%) | | | | |
|---|---|---|---|---|---|
| | Milk protein-Vit A complexes | | | | Free vitamin A (oily form) |
| | NaCas-VA ST | SNaCas-VA ST | RNaCas-VA | RSNaCas-VA | |
| **1** | 1.62±0.04$^{fB}$ | 1.70±0.01$^{fC}$ | 1.91±0.03$^{fD}$ | 2.53±0.10$^{fE}$ | 1.12±0.08$^{fA}$ |
| **2** | 1.28±0.05$^{eB}$ | 1.33±0.04$^{eB}$ | 1.48±0.11$^{eC}$ | 1.57±0.13$^{eD}$ | 0.59±0.09$^{eA}$ |
| **3** | 0.80±0.03$^{dB}$ | 1.05±0.06$^{dC}$ | 1.07±0.10$^{dC}$ | 1.12±0.08$^{dC}$ | 0.17±0.04$^{dA}$ |
| **4** | 0.62±0.06$^{cB}$ | 0.86±0.05$^{cC}$ | 0.97±0.09$^{cD}$ | 1.01±0.03$^{cD}$ | 0.09±0.01$^{cA}$ |
| **5** | 0.42±0.01$^{bB}$ | 0.74±0.04$^{bC}$ | 0.77±0.08$^{bC}$ | 0.85±0.03$^{bD}$ | 0.03±0.01$^{bA}$ |
| **6** | 0.17±0.01$^{aB}$ | 0.30±0.03$^{aC}$ | 0.60±0.02$^{aD}$ | 0.79±0.02$^{aE}$ | 0.02±0.00$^{aA}$ |

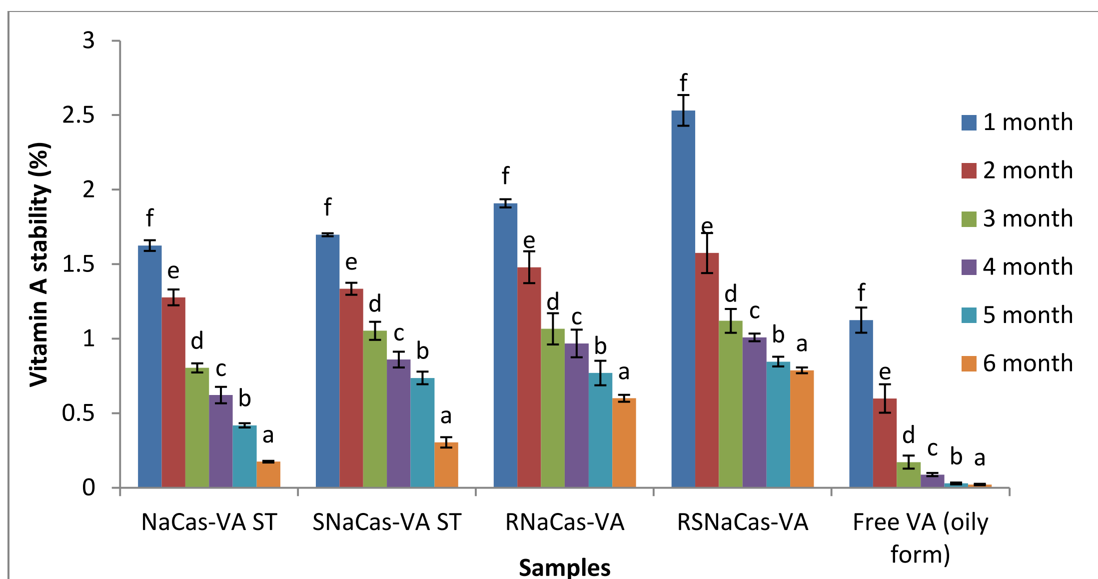
Data are presented as means±SEM (n=3)
$^{a-b}$Means within columns with different superscript are significantly different (p<0.05) from each other.
$^{A-B}$Means within rows with different superscript are significantly different (p<0.05) from each other.

storage and 0.02, 0.17, 0.30, 0.60 and 0.79% vitamin A stability after six months of storage. Vitamin A stability in all milk protein-Vit A complexes and free vitamin A (oily form) was significantly different (p<0.05) from each other during the storage period of six months. At 37°C, vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) followed similar trend as reported at -20 and 4°C. All milk protein-Vit A complexes and free vitamin A (oily form) showed significant decrease (p<0.05) in vitamin A stability during the storage period of six months.




$^{A-B}$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.

**Fig 4.39: Comparison of vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) stored in microcentrifuge tubes (polypropylene) at 37°C**




[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.

**Fig 4.40: Vitamin A stability in milk protein-Vit A complexes and free vitamin A (oily form) as affected by storage in microcentrifuge tubes (polypropylene) at 37°C**

RSNaCas-VA and RNaCas-VA showed higher stability may be due to the binding of vitamin A in the hydrophobic core (Table 4.24, 4.26, 4.28, 4.30, 4.32 and 4.34). Haham *et al.* (2012) also reported the binding of vitamin $D_3$ in the hydrophobic core of reassembled casein micelles.

Milk protein-Vit A complexes and free vitamin A (oily form) stored in aluminium laminate pouches showed lowest vitamin A stability at 37°C followed by 4°C and -20°C (Table 4.24, 4.26 and 4.28). However, milk protein-Vit A complexes and free vitamin A (oily form) showed lower vitamin A stability in microcentrifuge tubes as compared to aluminium laminate pouches (Table 4.24, 4.26, 4.28, 4.30, 4.32 and 4.34). Vitamin A was very less stable when stored in microcentrifuge tubes may be due to the presence of oxygen or easy permeability of oxygen. Our results are in accordance to Chavez-Servin *et al.* (2008b) who reported that vitamin A stability is affected by environmental conditions. Infant formulae exposed to light and oxygen are more susceptible to oxidative reaction and vitamin losses. Cladman *et al.* (1998) also reported that vitamin A was very less stable at 37°C which indicated that 37°C is inappropriate temperature for storage of vitamin A.

In our results binding of vitamin to milk protein increases the stability of vitamin A. Several researchers reported the similar findings. Zimet *et al.* (2011) reported that both docosahexaenoic acid (DHA) loaded reassembled casein micelles and DHA loaded casein nanoparticles showed remarkable protective effect against oxidation. They also reported several mechanism for protection of DHA from oxidation i.e. immobilization of fatty acid onto the protein, which decreases its mobility and consequently its chemical reactivity. Second is stearic shielding against penetration of oxidizing agents. Another shielding effect is against UV-light induced degradation as protein can absorb and scatter light and prevent it from reaching the DHA and improve its stability. Lastly, casein also has anti-oxidative and free radical quenching capacity which helps in improving the stability of DHA. Diarrassouba *et al.* (2014) reported that binding of ß-lg to vitamin $D_3$ improved the stability and extended the shelf life of vitamin $D_3$ at refrigerated storage conditions may be due the strong binding affinity of ß-lg for vitamin $D_3$. Complexation also improved the protection of vitamin $D_3$ against UV-light degradation and intestinal conditions. Diarrassouba *et al.* (2015) also reported that binding of vitamin $D_3$ to ß-lg based coagulum improved the stability of vitamin $D_3$ at 4°C and under UV-light exposure. Saiz-Abajo *et al.* (2013) reported that binding of ß-carotene in reassembled casein micelles improved the stability of ß-carotene for different thermal treatments i.e. pasteurization, sterilization, high hydrostatic pressure and baking.

Haham *et al.* (2012) reported that homogenized vitamin $D_3$-reassembled casein micelles and unhomogenized vitamin $D_3$-reassembled casein micelles showed higher stability after heat treatment and during cold storage as compared to vitamin $D_3$ in water and vitamin $D_3$ in tween-80 emulsion. Homogenized vitamin $D_3$-reassembled casein micelles showed higher stability during cold storage due to the disruption of large vitamin aggregates into smaller particles and improved coverage of vitamin droplets by caseins. Ron *et al.* (2010) also reported that binding of vitamin $D_2$ to ß-lg and ß-lg-pectin complex improved the stability of vitamin $D_2$ at room temperature for storage period of one week. Liang *et al.* (2011) also reported that binding of α-tocopherol to ß-lg improved the stability of α-tocopherol. Abbasi *et al.* (2014) reported that binding of vitamin $D_3$ to whey protein isolate and denatured whey protein isolate improved the stability of vitamin $D_3$ at room temperature for storage period of one week.

### 4.9.3 Evaluation of the effect of pH on stability of milk protein–Vit A complexes

The complex was dissolved in water and pH was adjusted to 3.0, 5.0 and 7.0. Sample was stored for 24 h and then centrifuged at 2236 g for 30 min (section 3.4.10). Stability of milk protein-Vit A complexes under different pH (3.0, 5.0 and 7.0) was analysed in terms of content (%) of vitamin A and protein in the supernatant. Upon change in pH, protein and vitamin tend to precipitate and it was assumed that if the content (%) of vitamin decreases (precipitation occurs) in the supernatant along with the decrease in protein content, then it was evident that the vitamin was bound to the protein in the form of a complex and vice versa. However, if the content of vitamin increases (precipitation does not occur) along with a decrease in protein content, then it was inferred that vitamin had separated from the complex i.e. stability of the complex was lost.

#### 4.9.3.1 Evaluation of the effect of pH 7.0 on stability of milk protein–Vit A complexes

Vitamin A and protein content (%) in the supernatant of NaCas-VA ST, RNaCas-VA and succinylated milk protein-Vit A complexes were significantly different ($p<0.05$) from each other (Table 4.35 and Fig 4.41). RNaCas-VA complex showed lowest vitamin A and protein content followed by NaCas-VA ST and succinylated milk protein-Vit A complexes (SNaCas-VA ST and RSNaCas-VA). Vitamin A and protein content in the supernatant of SNaCas-VA ST and RSNaCas-VA complexes were non significantly

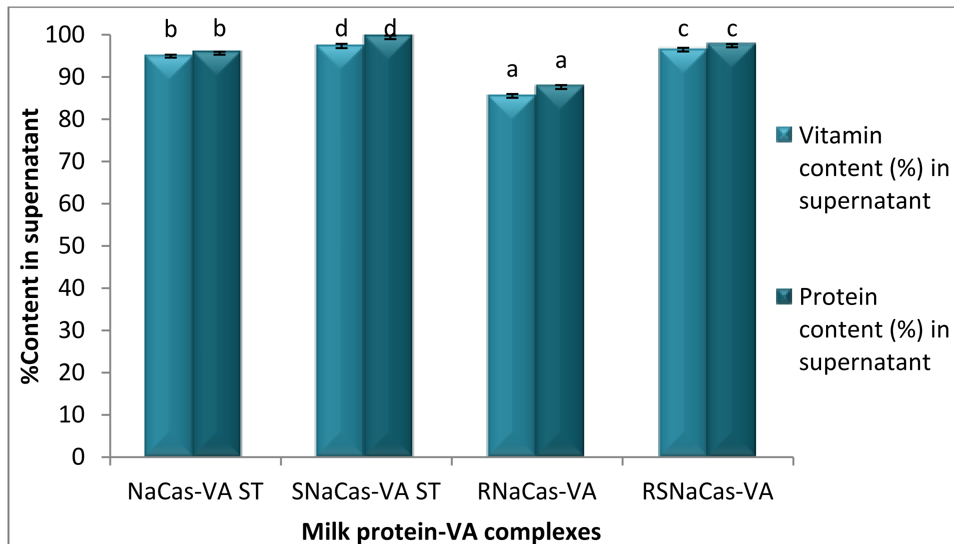
**Table 4.35: Content of vitamin A and protein (%) in the supernatant at pH 7.0**

| Samples | Vitamin A content (%) in supernatant | Protein content (%) in supernatant |
|---|---|---|
| **NaCas-VA ST** | $94.95\pm0.35^{b}$ | $95.63\pm0.35^{b}$ |
| **SNaCas-VA ST** | $97.36\pm0.51^{c}$ | $99.40\pm0.41^{c}$ |
| **RNaCas-VA** | $85.54\pm0.46^{a}$ | $87.64\pm0.47^{a}$ |
| **RSNaCas-VA** | $96.49\pm0.44^{c}$ | $97.43\pm0.38^{c}$ |

Data are presented as means±SEM (n=3)
$^{a-b}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.

different ($p>0.05$) from each other. All milk protein-Vit A complexes showed high protein and vitamin A content in the supernatant which indicated that vitamin A remained bound to milk protein at pH 7.0. It can be concluded from the results that all the milk protein-Vit

A complexes were stable at pH 7.0, hence, these complexes can be used as a fortificant in food products falling in pH range of 7.0.




[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.

**Fig 4.41: Content of vitamin A and protein (%) in the supernatant at pH 7.0**

### 4.9.3.2 Evaluation of the effect of pH 5.0 on stability of milk protein–Vit A complexes

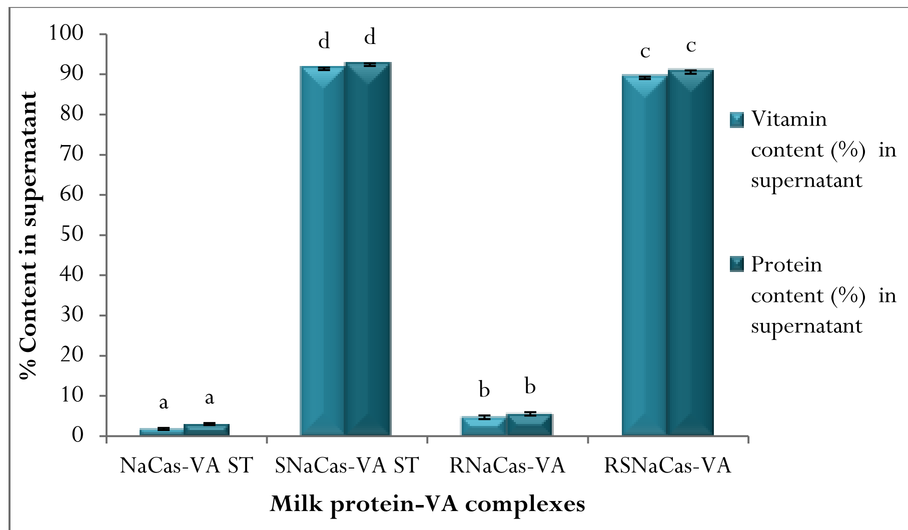
Vitamin A and protein content (%) in the supernatant of all the milk protein-Vit A complexes were significantly different ($p<0.05$) from each other (Table 4.36 and Fig 4.42). NaCas-VA ST complex showed lowest vitamin A and protein content in the supernatant followed by RNaCas-VA, RSNaCas-VA and SNaCas-VA ST complexes. Succinylated milk protein-Vit A complexes (SNaCas-VA ST and RSNaCas-VA) showed higher content of both vitamin A and protein in the supernatant, however, NaCas-VA ST and RNaCas-VA complexes showed lower content of both vitamin A and protein in the supernatant at pH 5.0. Protein content in the supernatant was directly related with vitamin A content in the supernatant which indicated that the binding between vitamin A and milk protein was not affected at pH 5.0. SNaCas-VA ST and RSNaCas-VA complexes showed high solubility at pH 5.0, hence, these complexes are suitable for use as a fortificant in food products i.e. whey beverages, acidic beverages, fruit juices, fruit pulp & concentrate etc. having a pH of 5.0. NaCas-VA ST and RNaCas-VA complexes showed poor solubility at pH 5.0. Since, protein undergoes precipitation at

**Table 4.36: Content of vitamin A and protein (%) in the supernatant at pH 5.0**

| Samples | Vitamin A content (%)in supernatant | Protein content (%) in supernatant |
|---|---|---|
| **NaCas-VA ST** | 1.74±0.26[a] | 2.96±0.25[a] |
| **SNaCas-VA ST** | 91.33±0.30[d] | 92.37±0.33[d] |
| **RNaCas-VA** | 4.65±0.47[b] | 5.50±0.43[b] |
| **RSNaCas-VA** | 89.09±0.30[c] | 90.53±0.42[c] |

Data are presented as means±SEM (n=3)
[a-b]Means within columns with different superscript are significantly different ($p<0.05$) from each other.

pH 5.0 and it alters the properties of milk protein-Vit A complexes, these complexes are not suitable for use as a fortificant in food products i.e. whey beverages, acidic beverages, fruit juices, fruit pulp & concentrate etc. having a pH of 5.0.




[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.

**Fig 4.42: Content of vitamin A and protein (%) in the supernatant at pH 5.0**

Succinylated milk protein-Vit A complexes showed higher protein content in supernatant, this may be due the modification in the isoelectric point of succinylated milk protein. Our results are in accordance to Franzen and Kinsella (1976) who reported that succinylation of soy protein shifted the iso electric point of protein from 4.5 to 4.0. Adebowale *et al.* (2009) reported that acetylation and succinylation of African yam bean protein isolate reduced protein solubility in the acidic pH range below the isoelectric point (4.5) of the protein concentrate, but improved the solubility of the unmodified protein concentrate at the isoelectric point and pH range alkaline to the isoelectric point.

Succinylation of lysozyme shifted the isoelectric point of protein from 11.0 to 4.6 (Van der Veen *et al.* 2004).

At low pH, hydrophobicity of casein increased and vitamin A may not be released from the milk protein-Vit A complexes. Liu and Guo (2008) reported that at pH 5.5, where the pH is near to the theoretical pI values of casein, the net charge of casein molecules is almost zero. Thus, the structure of the casein micelle is the most compact, and the Trp residues are located at the most hydrophobic domain. Compact structure of casein is due to the hydrophobic interaction between the different casein molecules resulting in coagulation of casein at isoelectric pH. Bringe and Kinsella (1987) stated that hydrophobic interactions are the main driving force for interactions between proteins for coagulation, gelation and gel synersis. Bringe and Kinsella (1991) also reported that displacement of $Ca^{2+}$, $Mg^{2+}$ or $Na^{+}$ with $H^{+}$ reduces the repulsive hydration forces between casein micelles and allows attractive hydration forces (e.g. hydrophobic phenomena) leading to casein coagulation.

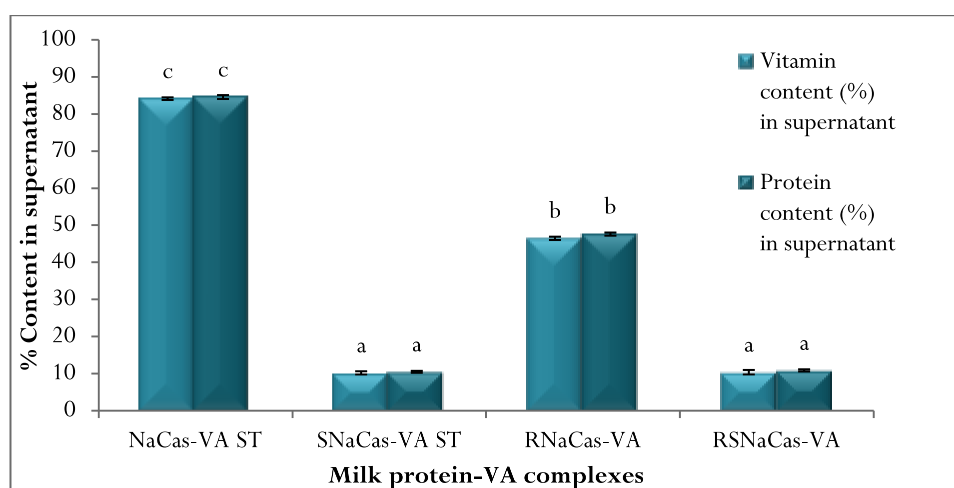
#### 4.9.3.3 Evaluation of the effect of pH 3.0 on stability of milk protein–Vit A complexes

Vitamin A and protein content in the supernatant of NaCas-VA ST, RNaCas-VA and succinylated milk protein-Vit A complexes were significantly different ($p<0.05$) from each other (Table 4.37 and Fig 4.43). Vitamin A and protein content in the supernatant of SNaCas-VA ST and RSNaCas-VA complexes were non significantly different ($p>0.05$) from each other. SNaCas-VA ST and RSNaCas-VA complexes showed lower vitamin A and protein content in the supernatant followed by RNaCas-VA and NaCas-VA ST complexes which indicated that the binding between vitamin A and milk protein remain intact at low pH as protein and vitamin content in the supernatant are directly related to each other. RNaCas-VA, SNaCas-VA ST and RSNaCas-VA complexes showed poor solubility at pH 3.0, however, NaCas-VA ST complex showed comparatively better solubility. Therefore, NaCas-VA ST complex was suitable to be use as fortificant, however, other milk protein-Vit A complexes i.e. SNaCas-VA ST, RNaCas-VA and RSNaCas-VA were not suitable to be used as fortificant in food products i.e. acidic beverages, fruit juices, fruit pulp & concentrate etc. having a pH of 3.0.

**Table 4.37: Content of vitamin A and protein (%) in the supernatant at pH 3.0**

| Samples | Vitamin A content (%) in supernatant | Protein content (%) in supernatant |
|---|---|---|
| **NaCas-VA ST** | 84.14±0.37[c] | 84.58±0.53[c] |
| **SNaCas-VA ST** | 10.18±0.44[a] | 10.47±0.26[a] |
| **RNaCas-VA** | 46.47±0.45[b] | 47.61±0.43[b] |
| **RSNaCas-VA** | 10.37±0.61[a] | 10.85±0.30[a] |

Data are presented as means±SEM (n=3)
[a-b]Means within columns with different superscript are significantly different (p<0.05) from each other.




[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.

**Fig 4.43: Content of vitamin A and protein (%) in the supernatant at pH 3.0**

Below pH 3.0, many amino acid residues in casein are effectively protonated. Since the ability of $H^+$ in $–NH_3^+$ to form hydrogen bond is much stronger than that of the neutral H atom in $–NH_2$, there exists strong hydrogen bond between casein molecules (Ma and Dougherty 1997). Below pH 3.0, casein molecules are stabilized by net positive charge on the casein micelles. Although, all calcium phosphate nanoclusters are mainly solubilised below pH 3.0, the mean size of casein micelles remains almost unchanged compared with that at pH 7.0. It indicated that the hydrogen bond together with the hydrophobic interaction is involved in the formation of casein micelles at acidic pH. Below pH 3.0, hydrophobic microdomains are tightly packed (Liu and Guo 2008), hence, vitamin A was not released from milk protein-Vit A complexes.

### 4.9.4 *In-vitro* bioavailability of vitamin A from milk protein-Vit A complexes and free vitamin A (oily form)

*In-vitro* bioavailability of vitamin A was lowest for free vitamin A (oily form) followed by NaCas-VA ST, SNaCas-VA ST, RNaCas-VA and RSNaCas-VA complexes (Table 4.38). RSNaCas-VA complex showed highest *in-vitro* bioavailability of vitamin A. All milk protein-Vit A complexes showed significantly higher ($p<0.05$) *in-vitro* bioavailability of vitamin A as compared to free vitamin A (oily form).

Binding of free vitamin A (oily form) to milk protein improved the *in-vitro* bioavailability of vitamin A. Our results were in accordance with Diarrassouba *et al.* (2014) who reported that binding of vitamin $D_3$ to ß-lg improved vitamin $D_3$ stability in simulated intestinal conditions and further improved its *in-vitro* bioavailability. Rats fed on these complexes showed higher level of 25-hydroxy-vitamin $D_3$ in plasma as compared to rats fed with free vitamin $D_3$ during *in-vivo* trials. Diarrassouba *et al.* (2015) reported binding of vitamin $D_3$ in ß-lg based coagulum and found that this complex was not rapidly disrupted by intestinal proteases, leading to higher stability of vitamin $D_3$ and subsequent enhancement of bioavailability of vitamin $D_3$ in rats. Teng *et al.* (2013) reported that vitamins microencapsulated with polysaccharides exhibited reduced release of vitamins under simulated intestinal conditions as compared to protein-vitamin complexes.

**Table 4.38: *In-vitro* bioavailability of vitamin A from milk protein-Vit A complexes and free vitamin A (oily form)**

| Samples | *In-vitro* bioavailability (%) |
|---|---|
| Free vitamin A (oily form) | $55.27\pm0.57^{a}$ |
| NaCas-VA ST | $73.29\pm0.52^{b}$ |
| SNaCas-VA ST | $73.41\pm0.48^{b}$ |
| RNaCas-VA | $74.44\pm0.56^{bc}$ |
| RSNaCas-VA | $75.75\pm0.58^{c}$ |

Data are presented as means±SEM (n=3)
$^{a-b}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.

### 4.10 Evaluation of sensory and physicochemical attributes of milk protein-Vit A complexes and free vitamin A (oily form) fortified milk

#### 4.10.1 Selection of milk protein-Vit A complexes for fortification of milk

Milk protein-Vit A complexes for milk fortification were selected on the basis of storage and pH stability. Storage stability of RSNaCas-VA and RNaCas-VA were significantly higher ($p<0.05$) than NaCas-VA ST and SNaCas-VA ST at -20, 4 and 37°C (Table 4.24, 4.26, 4.28, 4.30, 4.32 and 4.34). Among succinylated milk protein-Vit A complexes, RSNaCas-VA complex showed higher storage stability, however, SNaCas-VA and RSNaCas-VA complexes showed non significant difference ($p>0.05$) in solubility at pH 7.0 (Table 4.35). Hence, RSNaCas-VA complex was selected for the fortification of vitamin A in milk.

Among native milk protein-Vit A complexes, RNaCas-VA showed higher storage stability and lower solubility at pH 7.0, however, NaCas-VA ST showed lower storage stability and higher solubility at pH 7.0 (Table 4.35), hence, both complexes were selected for the fortification of milk. NaCas-VA ST, RNaCas-VA and RSNaCas-VA complexes were selected for fortification of vitamin A in milk.

#### 4.10.2 Effect of vitamin A fortification on sensory evaluation of milk

Sensory evaluation was carried out by a panel of ten trained judges who graded control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas- VA, RSNaCas-VA and free vitamin A (oily form)) milk for any change in colour and appearance, odour, taste and mouthfeel. Total sensory scores of control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas- VA, RSNaCas-VA and free vitamin A (oily form)) milk were non significantly different ($p>0.05$) from each other. Statistically, control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas- VA, RSNaCas-VA and free vitamin A (oily form)) milk did not differ significantly ($p>0.05$) in terms of colour and appearance, odour, taste and mouthfeel (Table 4.39). No change was observed by the sensory panel in natural flavour and odour of milk upon fortification. Our results were in accordance with Sachdeva *et al.* (2015) who reported that fortification of milk with vitamin A (2500 IU/L) and iron salt (25 ppm) does not have any significant ($p>0.05$) influence on sensory properties of milk. Kaushik *et al.* (2015a) also reported that fortification of milk with vitamin D (600 IU/L)

**Table 4.39: Sensory evaluation of control (unfortified) and vitamin A fortified milk**

| Characteristics | Maximum score | Control (Unfortified) | Vitamin A fortified milk | | | |
|---|---|---|---|---|---|---|
| | | | Free vitamin A (oily form) | NaCas-VA ST | RNaCas-VA | RSNaCas-VA |
| **Colour and appearance** | 10 | 9.66±0.04[a] | 9.63±0.03[a] | 9.60±0.03[a] | 9.60±0.05[a] | 9.60±0.01[a] |
| **Odour** | 20 | 19.04±0.05[a] | 18.97±0.06[a] | 18.95±0.03[a] | 19.15±0.02[a] | 19.00±0.03[a] |
| **Taste** | 40 | 38.53±0.04[a] | 38.49±0.06[a] | 38.46±0.07[a] | 38.80±0.05[a] | 38.40±0.10[a] |
| **Mouthfeel** | 30 | 28.85±0.06[a] | 28.79±0.07[a] | 28.75±0.06[a] | 28.65±0.08[a] | 28.70±0.05[a] |
| **Total score** | 100 | 96.09±0.12[a] | 95.88±0.09[a] | 95.76±0.14[b] | 96.20±0.17[a] | 95.70±0.15[a] |

Data are presented as means±SEM (n=3)
[a-b]Means within columns with different superscript are significantly different ($p<0.05$) from each other.

and calcium salt (500 ppm) does not have any significant ($p>0.05$) influence on sensory properties of milk.

### 4.10.3 Effect of vitamin A fortification on alcohol stability of milk

The freshness of milk and its suitability for UHT processing can be indicated by alcohol (ethanol) stability test (Shew 1981). Milk, stable in 74% alcohol is suitable for UHT treatment (Boumpa *et al*. 2008). Effect of vitamin A fortification on ethanol stability of milk is presented in table 4.40. Control (unfortified) and vitamin A fortified milk (containing NaCas-VA ST, RNaCas- VA, RSNaCas-VA and free vitamin A (oily form)) 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 of UHT treated dairy products.

**Table 4.40: Effect of vitamin A fortification on alcohol stability of milk**

| Sample | | Result |
|---|---|---|
| **Control (unfortified) milk** | | Alcohol negative |
| **Vitamin A fortified milk** | **Free vitamin A (oily form)** | Alcohol negative |
| | **NaCas-VA ST** | Alcohol negative |
| | **RNaCas-VA** | Alcohol negative |
| | **RSNaCas-VA** | Alcohol negative |

### 4.10.4 Effect of vitamin A fortification on pH and titratable acidity of milk

pH and titratable acidity of control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas- VA, RSNaCas-VA and free vitamin A (oily form)) milk are presented in table 4.41. pH and titratable acidity of control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas- VA, RSNaCas-VA and free vitamin A (oily form)) milk samples did not differ significantly ($p>0.05$) from each other. Our results were in accordance with Sachdeva *et al.* (2015) who reported that fortification of milk with vitamin A (2500 IU/L) and iron salt (25 ppm) does not have any significant ($p>0.05$) influence on pH and titratable acidity. Kaushik *et al.* (2015a) also reported that fortification of milk with vitamin D (600 IU/L) and calcium salt (500 ppm) does not have any significant ($p>0.05$) influence on pH and titratable acidity.

**Table 4.41: Effect of vitamin A fortification on pH and titratable acidity of milk**

| Sample | | pH | Titratable acidity (%LA) |
|---|---|---|---|
| **Control (unfortified) milk** | | 6.65±0.009$^{a}$ | 0.141±0.003$^{a}$ |
| **Vitamin A fortified milk** | **Free vitamin A (oily form)** | 6.66±0.006$^{a}$ | 0.148±0.004$^{a}$ |
| | **NaCas-VA ST** | 6.66±0.003$^{a}$ | 0.141±0.003$^{a}$ |
| | **RNaCas-VA** | 6.67±0.006$^{a}$ | 0.148±0.004$^{a}$ |
| | **RSNaCas-VA** | 6.67±0.009$^{a}$ | 0.141±0.003$^{a}$ |

Data are presented as means±SEM (n=3)
$^{a-b}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.

### 4.10.5 Effect of vitamin A fortification on heat stability of milk

Heat stability of evaporated milk refers to the relative resistance of milk to coagulation in the sterilizer. It is also defined as “the time necessary to initiate coagulation at 120°C”. It is important in the processing operation of concentrated product manufacture. 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 (Kaushik *et al.* 2015b).

#### 4.10.5.1 Heat stability of control (unfortified) milk

The heat stability (as HCT) of control milk at its natural pH and after adjustment of the pH between 6.4 to 7.0 at 0.1 unit intervals using 0.1 N NaOH and

0.1 N HCl was studied and the results are shown in table 4.42 and figure 4.44. The results revealed that HCT of toned milk (cow milk:buffalo milk 1:1) at 140±1°C at its natural pH (6.66) was 25.73 min

HCT ranged from 36.68 min (at pH 6.5) to 2.41 min (at pH 7.0) and the maxima in the HCT/pH curve (at pH 6.5: 36.68 min) was on the acidic side of natural pH of milk. (pH 6.66). HCT at pH 6.4 was 32.04 min and there was a rise in the HCT at pH 6.5 (36.68 min). Further increase in pH above 6.5 caused a gradual decrease in HCT upto pH 6.7 followed by a steep decline thereafter.

**Table 4.42: Heat stability of control (unfortified) toned milk**

| pH→ Samples↓ | HCT (in min) at 140ºC | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Ctrl Milk pH | pH adjusted with 0.1 N NaOH and 0.1 N HCl | | | | | | |
| | 6.66 ±0.02 | 6.4 | 6.5 | 6.6 | 6.7 | 6.8 | 6.9 | 7.0 |
| 1. | 26.57 | 32.25 | 36.44 | 35.26 | 24.5 | 3.43 | 3.15 | 2.26 |
| 2. | 24.1 | 32.05 | 37.06 | 34.5 | 22.45 | 5.1 | 4.1 | 2.45 |
| 3. | 26.51 | 31.82 | 36.54 | 36.15 | 25.57 | 5.15 | 4.18 | 2.54 |
| **Average** | 25.73± 0.81 | 32.04± 0.12 | 36.68± 0.19 | 35.30± 0.48 | 24.17± 0.92 | 4.56±0.57 | 3.81±0.33 | 2.41±0.08 |

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

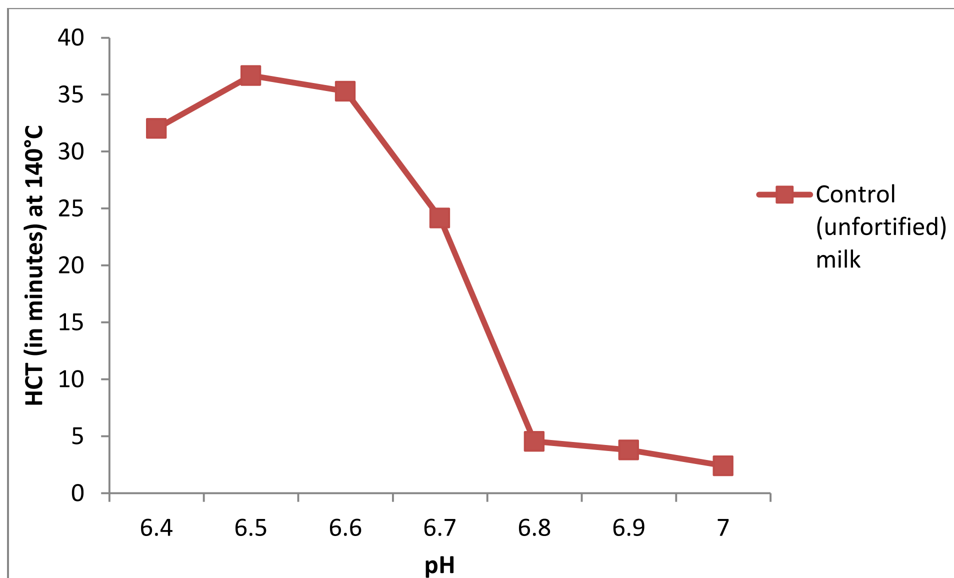



**Fig 4.44: HCT/pH curve of control (unfortified) milk**

### 4.10.5.2 Heat stability of free vitamin A (oily form) fortified milk

Heat stability of free vitamin A (oily form) fortified milk (@2000 IU/L of vitamin A) at its natural pH and after adjustment of the pH between 6.4 to 7.0 at 0.1 unit intervals using 0.1 N NaOH and 0.1 N HCl was studied and the results are shown in table 4.43 and figure 4.45. The results revealed that HCT of control (unfortified) milk at 140±1°C at its natural pH (6.66) was 25.73 min and that of free vitamin A (oily form) fortified milk (pH 6.66) was 25.93 min i.e. negligible difference was observed between the HCT of control milk and fortified milk at their natural pH. HCT of fortified milk ranged from 37.57 min (pH 6.5) to 5.16 min (at pH 7.0) and the maxima in HCT/pH curve (at pH 6.5: 37.57 min) was on the acidic side of natural pH of fortified milk (pH 6.66). HCT at pH 6.4 was 33.95 min and there was a rise in the HCT at pH 6.5 (37.57 min). Further increase in pH above 6.5 caused a gradual decrease in HCT upto pH 6.7 followed by a steep decline thereafter. Since, control and vitamin A fortified milk sample were showing comparable HCT at their natural pH, therefore, it can be concluded that addition of free vitamin A (oily form) @2000 IU/L did not affect the heat stability of milk.

**Table 4.43: Heat stability of free vitamin A (oily form) fortified milk**

| pH→ Samples↓ | HCT (in min) at 140ºC | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Ctrl milk pH | Free vitamin A (oily form) fortified milk (2000 IU/L of vitamin A) | | | | | | | |
| | | Fortd milk pH | pH adjusted with 0.1 N NaOH and 0.1 N HCl | | | | | | |
| | **6.66 ±0.02** | **6.66 ±0.02** | **6.4** | **6.5** | **6.6** | **6.7** | **6.8** | **6.9** | **7.0** |
| 1. | 26.57 | 25.48 | 35.06 | 39.15 | 36.1 | 21.25 | 7.35 | 6.5 | 6 |
| 2. | 24.1 | 24.25 | 32.24 | 35.38 | 35.2 | 20.5 | 5.25 | 5 | 4.4 |
| 3. | 26.51 | 28.06 | 34.56 | 38.18 | 37.18 | 23.68 | 6.57 | 7.03 | 5.09 |
| **Average** | 25.73± 0.81 | 25.93± 1.12 | 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).

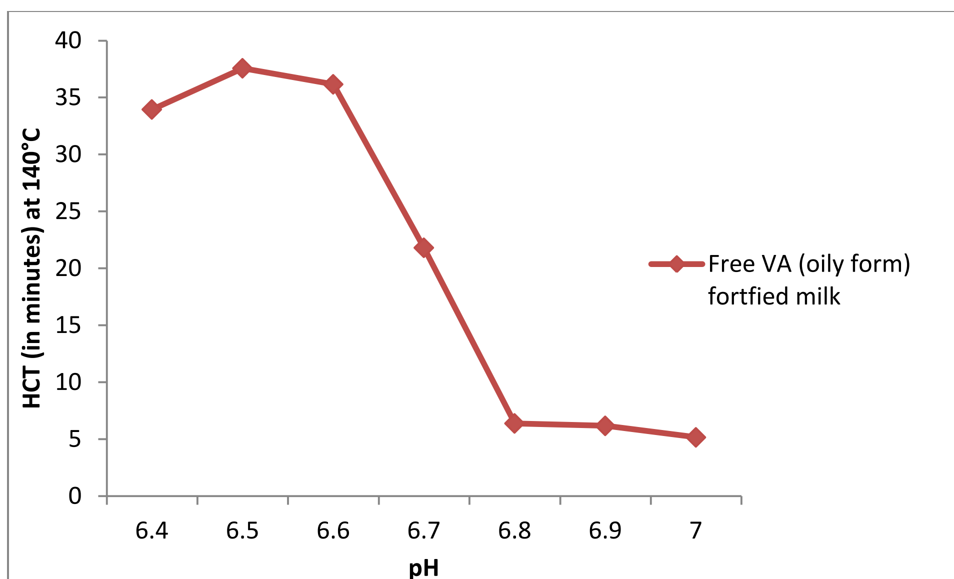



**Fig 4.45: HCT/pH curve of free vitamin A (oily form) (@2000 IU/L of vitamin A) fortified milk**

### 4.10.5.3 Heat stability of NaCas-VA ST fortified milk

Heat stability of NaCas-VA ST fortified milk (@2000 IU/L of vitamin A) at its natural pH and after adjustment of the pH between 6.4 to 7.0 at 0.1 unit intervals using 0.1 N NaOH and 0.1 N HCl was studied and the results are shown in table 4.44 and figure 4.46. The results revealed that HCT of control (unfortified) milk at 140±1°C at its natural pH (6.73) was 21.08 min and that of NaCas-VA ST fortified milk (pH 6.73) was 21.48 min i.e. negligible difference was observed in the HCT of control milk and fortified milk at their natural pH. HCT of fortified milk ranged from 37.22 min (pH 6.5) to 5.28 min (at pH 7.0) and the maxima in HCT/pH curve (at pH 6.5: 37.22 min) was on the acidic side of natural pH of fortified milk (pH 6.73). HCT at pH 6.4 was 32.58 min and there was a rise in the HCT at pH 6.5 (37.22 min). Further increase in pH above 6.5 caused a gradual decrease in HCT upto pH 6.7 followed by a steep decline thereafter. Since, control and vitamin A fortified milk sample were showing comparable HCT at their natural pH, therefore, it was concluded that addition of NaCas-VA ST @2000 IU/L of vitamin A did not affect the heat stability of milk.

**Table 4.44: Heat stability of NaCas-VA ST fortified milk**

| pH→ Samples↓ | HCT (in min) at 140ºC | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Ctrl milk pH | NaCas-VA ST fortified milk (2000 IU/L of vitamin A) | | | | | | | |
| | | Fortd milk pH | pH adjusted with 0.1 N NaOH and 0.1 N HCl | | | | | | |
| | 6.73 ±0.02 | 6.73 ±0.02 | 6.4 | 6.5 | 6.6 | 6.7 | 6.8 | 6.9 | 7.0 |
| 1. | 20.65 | 21.06 | 32.06 | 36.08 | 35.94 | 24.38 | 6.54 | 6.3 | 6.15 |
| 2. | 19.96 | 23.34 | 30.54 | 35.37 | 33.46 | 27.19 | 6.13 | 5.58 | 5.32 |
| 3. | 22.64 | 20.04 | 35.13 | 40.22 | 39.11 | 23.29 | 7.22 | 6.23 | 6 |
| **Average** | 21.08± 0.80 | 21.48± 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).

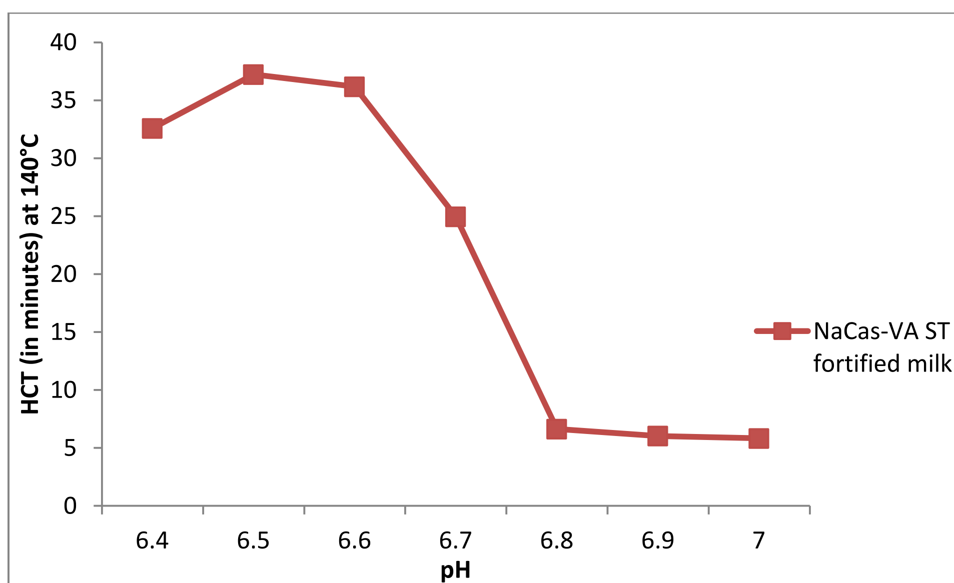



**Fig 4.46: HCT/pH curve of NaCas-VA ST (@2000 IU/L of vitamin A) fortified milk**

### 4.10.5.4 Heat stability of RNaCas-VA fortified milk

Heat stability of RNaCas-VA fortified milk (@2000 IU/L of vitamin A) at its natural pH and after adjustment of the pH between 6.4 to 7.0 at 0.1 unit intervals using 0.1 N NaOH and 0.1 N HCl was studied and the results are shown in table 4.45 and figure 4.47. The results revealed that HCT of control (unfortified) milk at

140±1°C at its natural pH (6.64) was 24.02 min and that of RNaCas-VA fortified milk (pH 6.64) was 23.62 min i.e. negligible difference was observed in the HCT of control milk and fortified milk at their natural pH. HCT of fortified milk ranged from 35.34 min (pH 6.5) to 3.40 min (at pH 7.0) and the maxima in HCT/pH curve (at pH 6.5: 35.34 min) was on the acidic side of natural pH of fortified milk (pH 6.64). HCT at pH 6.4 was 31.57 min and there was a rise in the HCT at pH 6.5 (35.34 min). Further increase in pH above 6.5 caused a gradual decrease in HCT upto pH 6.7 followed by a steep decline thereafter. Since, control and vitamin A fortified milk sample were showing comparable HCT at their natural pH, therefore, it can be concluded that addition of RNaCas-VA @2000 IU/L of vitamin A did not affect the heat stability of milk.

**Table 4.45: Heat stability of RNaCas-VA fortified milk**

| pH→ Samples↓ | HCT (in min) at 140ºC | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Ctrl milk pH | RNaCas-VA fortified milk (2000 IU/L of vitamin A) | | | | | | | |
| | | Fortd milk pH | pH adjusted with 0.1 N NaOH and 0.1 N HCl | | | | | | |
| | 6.64 ±0.02 | 6.64 ±0.02 | 6.4 | 6.5 | 6.6 | 6.7 | 6.8 | 6.9 | 7.0 |
| 1. | 24.81 | 23.06 | 31.36 | 34.18 | 36.08 | 21.47 | 5.94 | 3.48 | 2.58 |
| 2. | 23.76 | 22.58 | 33.19 | 38.33 | 36.08 | 20.41 | 7.26 | 7 | 5.35 |
| 3. | 23.49 | 25.23 | 30.15 | 33.51 | 32.02 | 21.18 | 5.41 | 5.04 | 2.27 |
| **Average** | 24.02± 0.40 | 23.62± 0.82 | 31.57± 0.88 | 35.34± 1.51 | 34.72± 1.35 | 21.02± 0.32 | 6.20±0. 55 | 5.17±1. 01 | 3.40±0 .98 |

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

### 4.10.5.5 Heat stability of RSNaCas-VA fortified milk

Heat stability of RSNaCas-VA fortified milk (@2000 IU/L of vitamin A) at its natural pH and after adjustment of the pH between 6.4 to 7.0 at 0.1 unit intervals using 0.1 N NaOH and 0.1 N HCl was studied and the results are shown in table 4.46 and figure 4.48. The results revealed that HCT of control (unfortified) milk at 140±1°C at its natural pH (6.66) was 26.91 min and that of RSNaCas-VA fortified

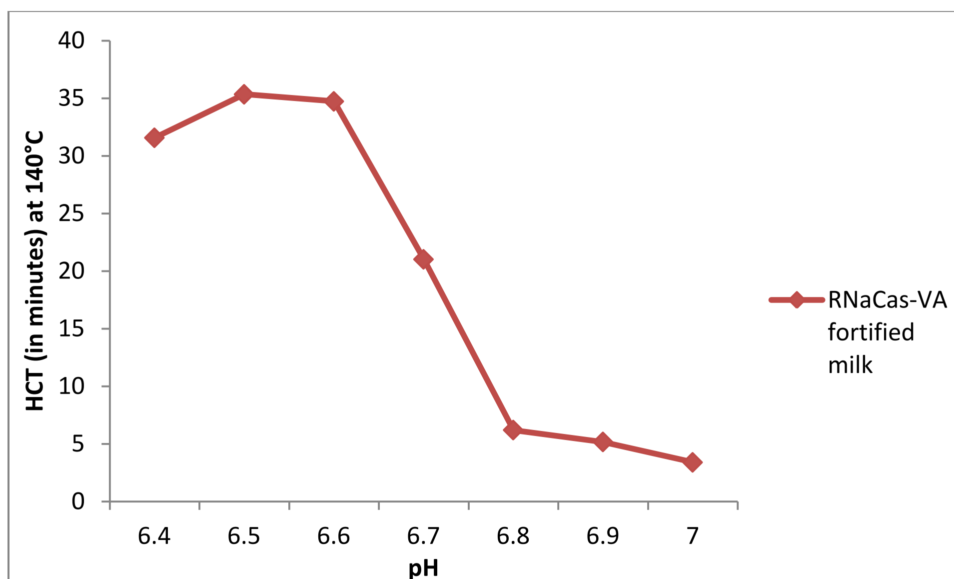



**Fig 4.47: HCT/pH curve of RNaCas-VA (@2000 IU/L of vitamin A) fortified milk**

milk (pH 6.66) was 26.64 min i.e. negligible difference was observed in the HCT of control milk and fortified milk at their natural pH. HCT of fortified milk ranged from 35.93 min (pH 6.5) to 3.05 min (at pH 7.0) and the maxima in HCT/pH curve (at pH 6.5: 35.93 min) was on the acidic side of natural pH of fortified milk (pH 6.66). HCT at pH 6.4 was 32.54 min and there was a rise in the HCT at pH 6.5 (35.93 min).

**Table 4.46: Heat stability of RSNaCas-VA fortified milk**

| pH→ Samples↓ | HCT (in min) at 140ºC | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Ctrl milk pH | RSNaCas-VA fortified milk (2000 IU/L of vitamin A) | | | | | | | |
| | | Fortd milk pH | pH adjusted with 0.1 N NaOH and 0.1 N HCl | | | | | | |
| | 6.66 ±0.02 | 6.66 ±0.02 | 6.4 | 6.5 | 6.6 | 6.7 | 6.8 | 6.9 | 7.0 |
| 1. | 25.78 | 30.05 | 30.4 | 34.54 | 34.04 | 27.48 | 5.3 | 4.1 | 3.35 |
| 2. | 26.98 | 22.4 | 33.09 | 36.78 | 36.27 | 28.53 | 3.45 | 2.45 | 2.3 |
| 3. | 27.98 | 27.48 | 34.13 | 36.47 | 35.87 | 26.94 | 4.3 | 4.15 | 3.5 |
| **Average** | 26.91± 0.64 | 26.64± 2.25 | 32.54± 1.11 | 35.93± 0.70 | 35.39± 0.69 | 27.65± 0.47 | 4.35±0.53 | 3.57±0.56 | 3.05±0.38 |

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

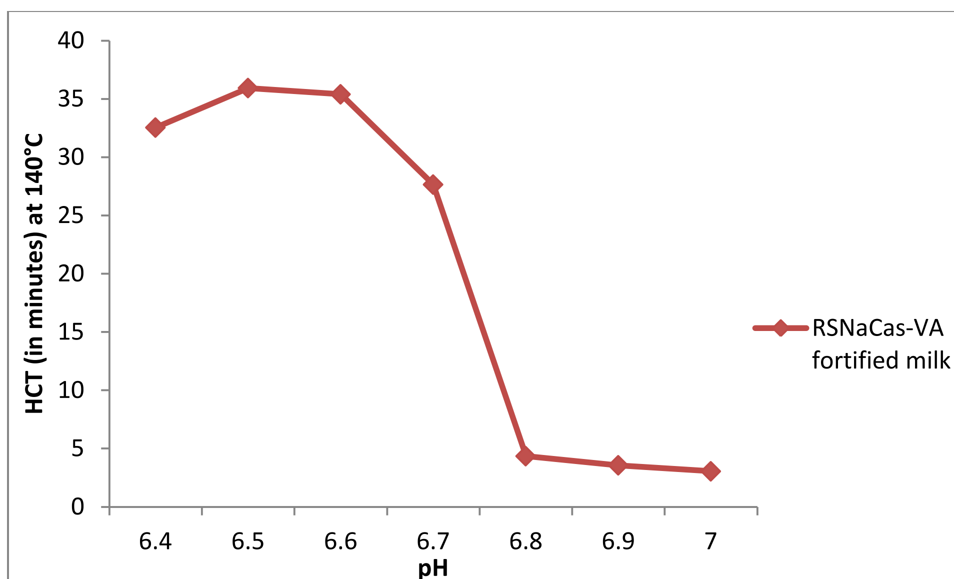



**Fig 4.48: HCT/pH curve of RSNaCas-VA (@2000 IU/L of vitamin A) fortified milk**

Further increase in pH above 6.5 caused a gradual decrease in HCT upto pH 6.7 followed by a steep decline thereafter. Since, control and vitamin A fortified milk sample were showing comparable HCT at their natural pH, therefore, it can be concluded that addition of RSNaCas-VA @2000 IU/L of vitamin A did not affect the heat stability of milk.

It was observed that HCT/pH curve of control (unfortified) and vitamin A fortified milk were comparable (Fig 4.49). Vitamin A fortification does not have an influence on heat stability of milk. The comparable heat stability of control (unfortified) and vitamin A fortified milk could be due to negligible change in pH caused by the addition of free vitamin A (oily form) and milk protein-Vit A complexes. It was evident from the figure 4.49 that control (unfortified) milk and vitamin A fortified milk (containing NaCas-VA ST, RNaCas- VA, RSNaCas-VA and free vitamin A (oily form)) exhibited type A characteristics with pH of maximum stability at acidic side of natural pH. Therefore, it can be concluded that fortification of milk with vitamin A did not cause any adverse change in milk system. Sachdeva *et al.* (2015) also reported that fortification of milk with ferric pyrophosphate soluble, ferrous gluconate hydrate and vitamin A did not affect the heat stability of milk.

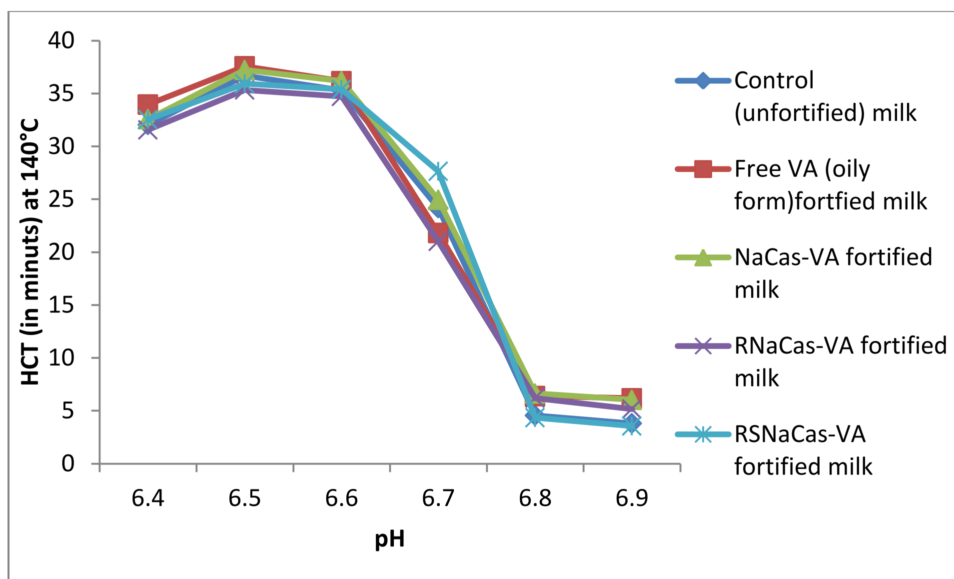



**Fig 4.49: Comparison of HCT/pH curve of control (unfortified) and vitamin A fortified milk samples**

### 4.10.6 Effect of vitamin A fortification on viscosity of milk

Viscosity of control (unfortified) and vitamin A fortified milk (containing NaCas-VA ST, RNaCas- VA, RSNaCas-VA and free vitamin A (oily form)) was measured at 27ºC using Ostwald's U-tube viscometer and the results are shown in table 4.47. There was no effect of fortification ($p>0.05$) of vitamin A (NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) on viscosity of milk and the values were similar with the control (unfortified) milk. Our results were in accordance with Bhawana (2012) who reported non significant effect ($p>0.05$) of vitamin A and iron fortification on viscosity of milk.

**Table 4.47: Effect of vitamin A fortification on viscosity of milk**

| Sample | | Viscosity (cP) |
|---|---|---|
| **Control (unfortified) milk** | | $1.60 \pm 0.02^{a}$ |
| **Vitamin A fortified milk** | **Free vitamin A (oily form)** | $1.63 \pm 0.03^{a}$ |
| | **NaCas-VA ST** | $1.63 \pm 0.02^{a}$ |
| | **RNaCas-VA** | $1.62 \pm 0.03^{a}$ |
| | **RSNaCas-VA** | $1.62 \pm 0.02^{a}$ |

Data are presented as means±SEM (n=3)
$^{a-b}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.

### 4.10.7 Effect of vitamin A fortification on curd tension and RCT of milk

Curd tension and RCT of control (unfortified) and vitamin A fortified milk (containing NaCas-VA ST, RNaCas- VA, RSNaCas-VA and free vitamin A (oily form)) did not differ significantly (p>0.05) from each other (Table 4.48). Vitamin A fortification does not influence the curd tension and RCT of milk. Our results are in accordance with Bhawana (2012) who reported non significant effect (p>0.05) of vitamin A and iron fortification on curd tension and RCT of milk.

**Table 4.48: Effect of vitamin A fortification on curd tension and RCT of milk**

| Sample | | Curd tension (g) | RCT (seconds) |
|---|---|---|---|
| Control (unfortified) milk | | 23.98±0.24[a] | 132.89±0.34[a] |
| Vitamin A fortified milk | Free vitamin A (oily form) | 23.35±0.22[a] | 131.40±0.09[a] |
| | NaCas-VA ST | 23.33±0.13[a] | 132.34±0.20[a] |
| | RNaCas-VA | 23.43±0.19[a] | 133.80±0.95[a] |
| | RSNaCas-VA | 23.67±0.17[a] | 133.94±0.08[a] |

Data are presented as means±SEM (n=3)
[a-b]Means within columns with different superscript are significantly different (p<0.05) from each other.

### 4.10.8 Effect of vitamin A fortification on colour profile of milk

Colour of control (unfortified) and vitamin A fortified milk (containing NaCas-VA ST, RNaCas- VA, RSNaCas-VA and free vitamin A (oily form)) was determined using 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).

**Table 4.49: Effect of vitamin A fortification on colour profile of milk**

| Sample | | L value | a value | b value |
|---|---|---|---|---|
| Control (unfortified) | | 87.42±0.04[a] | -2.61±0.02[a] | 7.60±0.02[a] |
| Vitamin A fortified milk | Free vitamin A (oily form) | 87.39±0.02[a] | -2.58±0.02[a] | 7.66±0.01[a] |
| | NaCas-VA ST | 87.34±0.07[a] | -2.55±0.01[a] | 7.57±0.01[a] |
| | RNaCas-VA | 87.33±0.01[a] | -2.48±0.04[a] | 7.59±0.02[a] |
| | RSNaCas-VA | 87.36±0.01[a] | -2.54±0.05[a] | 7.59±0.03[a] |

Data are presented as means±SEM (n=3)
[a-b]Means within columns with different superscript are significantly different (p<0.05) from each other.

Colourflex readings for control and vitamin A fortified milk are presented in the table 4.49. Fortification of milk with vitamin A did not significantly (p>0.05) effect the colour profile of milk. Control and vitamin A fortified milk samples showed non significant difference (p>0.05) in L, a and b value. No reports are available regarding the effect of vitamin A fortification on colour profile of milk.

## 4.11 Evaluation of the effect of different processing and storage conditions on stability of vitamin A in milk protein-Vit A complexes and free vitamin A (oily form) fortified milk

Toned milk was fortified with milk protein-Vit A complexes (NaCas-VA ST, RNaCas-VA and RSNaCas-VA) and free vitamin A (oily form). Control (unfortified) and vitamin A fortified milk was subjected to different heat treatments i.e. pasteurisation (63ºC/30 min), boiling and sterilisation (121ºC/15 min/15 psi) and stability of vitamin A was analysed after each heat treatment. Pasteurised milk was packed in LDPE pouches and transparent glass bottles and stored at refrigerated temperature (4-7°C). The vitamin A content was analysed on 0, 3$^{rd}$, 5$^{th}$ and 7$^{th}$ day of storage. Pasteurised milk packed in LDPE pouches and transparent glass bottles was also exposed to different light intensities (1485, 2970 and 4455 lux) and vitamin A content was estimated after 12, 24, 36 and 48 h of storage. Sterilised milk was packed in transparent glass bottles and stored at room temperature (37°C). Samples were analysed for vitamin A content at a time interval of 15 days upto 90 days of storage.

### 4.11.1 Evaluation of the effect of different heat treatment

Vitamin A stability is affected by severity of heat treatment i.e. more severe the heat treatment, lower will be the heat stability. Effect of heat treatment on vitamin A content of milk is presented in table 4.50 which indicated that vitamin A content was significantly affected (p<0.05) by different heat treatments. All heat treatments resulted in decrease in vitamin A content in all the milk samples. Vitamin A content was lowest in sterilized milk followed by boiled and pasteurized control (unfortified) milk. Similar trend was observed for the vitamin A fortified (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) milk samples.

**Table 4.50: Effect of heat treatment on vitamin A content of milk**

| Sample | | Vitamin A content (IU/L) | | | |
|---|---|---|---|---|---|
| | | Raw milk | Pasteurised milk | Boiled milk | Sterilised milk |
| Control (unfortified) | | 403.21±8.31$^{C}$ | 394.18±7.70$^{C}$ | 306.55±6.58$^{B}$ | 274.19±8.09$^{A}$ |
| Vitamin A fortified milk | Free vitamin A (oily form) | 2334.68±25.58$^{D}$ | 2121.51±29.59$^{C}$ | 1601.53±14.93$^{B}$ | 1447.48±22.39$^{A}$ |
| | NaCas-VA ST | 2502.32±16.65$^{D}$ | 2413.75±11.49$^{C}$ | 1900.62±20.59$^{B}$ | 1739.14±14.83$^{A}$ |
| | RNaCas-VA | 2470.15±18.53$^{D}$ | 2393.47±22.68$^{C}$ | 1910.84±11.20$^{B}$ | 1784.20±19.77$^{A}$ |
| | RSNaCas-VA | 2533.26±27.55$^{D}$ | 2455.40±32.00$^{C}$ | 1987.04±31.11$^{B}$ | 1848.46±25.47$^{A}$ |

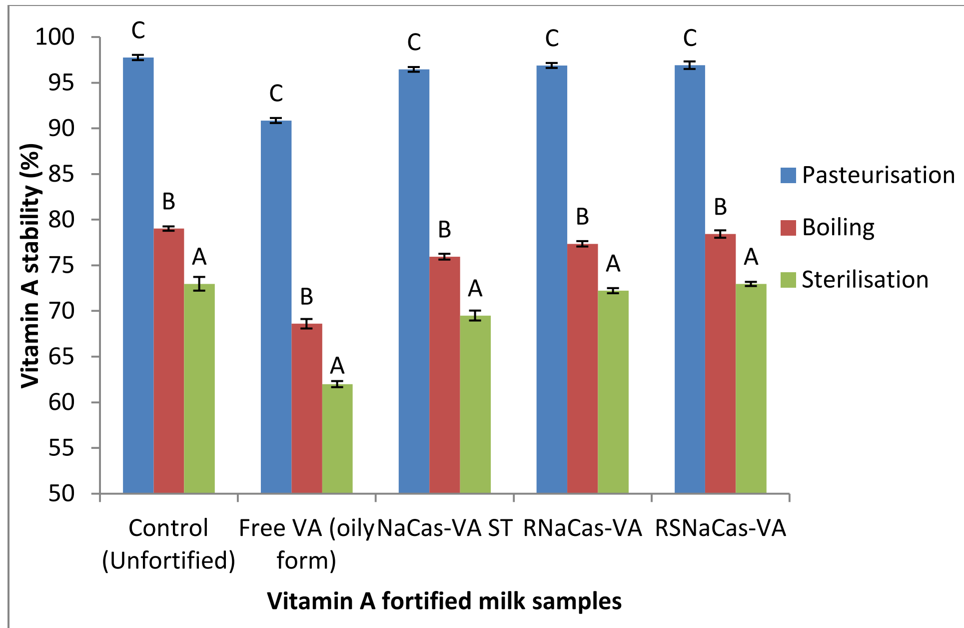
Data are presented as means±SEM (n=3)
$^{A-B}$Means within rows with different superscript are significantly different (p<0.05) from each other.

Effect of heat treatment on vitamin A stability is presented in table 4.51 which indicated that vitamin A stability was lowest after sterilization followed by boiling and pasteurization. Vitamin A stability of control (unfortified) and vitamin A fortified milk (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) samples was significantly affected ($p<0.05$) by different heat treatments (Fig 4.50). Pasteurisation significantly affected ($p<0.05$) the vitamin A stability in control (unfortified) and vitamin A fortified milk (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) samples (Table 4.51 and Fig 4.51).

**Table 4.51: Effect of heat treatment on vitamin A stability in milk**

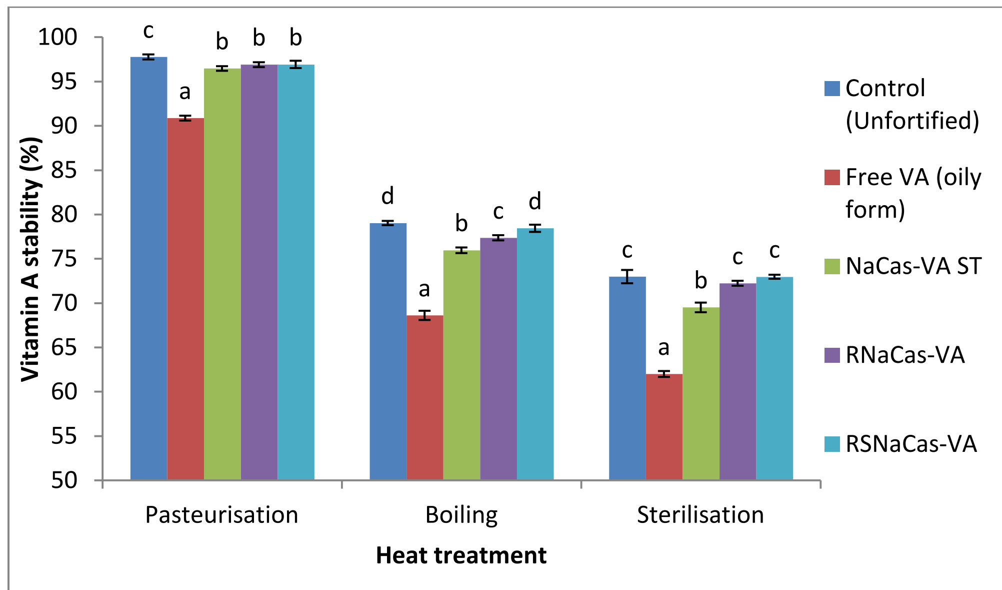
| Sample | | Vitamin A stability (%) | | |
|---|---|---|---|---|
| | | Pasteurised milk | Boiled milk | Sterilised milk |
| Control (unfortified) | | 97.77±0.29$^{cC}$ | 79.03±0.24$^{dB}$ | 72.98±0.75$^{cA}$ |
| Vitamin A fortified milk | Free vitamin A (oily form) | 90.86±0.27$^{aC}$ | 68.60±0.52$^{aB}$ | 61.99±0.33$^{aA}$ |
| | NaCas-VA ST | 96.46±0.26$^{bC}$ | 75.95±0.32$^{bB}$ | 69.50±0.54$^{bA}$ |
| | RNaCas-VA | 96.89±0.27$^{bC}$ | 77.36±0.29$^{cB}$ | 72.23±0.28$^{cA}$ |
| | RSNaCas-VA | 96.92±0.41$^{bC}$ | 78.43±0.41$^{dB}$ | 72.96±0.23$^{cA}$ |

Data are presented as means±SEM (n=3)
$^{a-b}$Means within columns with different superscript are significantly different (p<0.05) from each other.
$^{A-B}$Means within rows with different superscript are significantly different (p<0.05) from each other.




[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.

**Fig 4.50: Effect of different heat treatment on vitamin A stability**




[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.

**Fig 4.51: Comparison of vitamin A stability in control and vitamin A fortified milk samples as affected by different heat treatment**

After pasteurization, control (unfortified) milk showed the highest vitamin A stability followed by milk protein-Vit A complexes and free vitamin A (oily form) fortified milk. NaCas-VA ST, RNaCas-VA and RSNaCas-VA fortified milk showed non significant difference ($p>0.05$) in vitamin A stability. Free vitamin A (oily form) fortified milk showed the lowest stability of vitamin A.

Boiling significantly affected ($p<0.05$) the vitamin A stability in control (unfortified) and vitamin A fortified milk (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) samples (Table 4.51 and Fig 4.51). After boiling, free vitamin A (oily form) fortified milk showed the lowest vitamin A stability followed by NaCas-VA ST, RNaCas-VA, RSNaCas-VA fortified and control (unfortified) milk. Control (unfortified) and RSNaCas-VA fortified milk showed non significant difference ($p>0.05$) in vitamin A stability.

Sterilisation significantly affected ($p<0.05$) the vitamin A stability in control (unfortified) and vitamin A fortified milk (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) samples (Table 4.51 and Fig 4.51). After sterilisation, free vitamin A (oily form) fortified milk showed the lowest vitamin A stability followed by NaCas-VA ST, RNaCas-VA, RSNaCas-VA fortified and control (unfortified) milk.. RSNaCas-VA and RNaCas-VA showed higher stability may be due to the binding of vitamin A in the hydrophobic core as reported in the section 4.9.2. Haham *et al.* (2012) also reported the binding of vitamin $D_3$ in the hydrophobic core of reassembled casein micelles.

Bector and Rani (1998) observed that vitamin A content was not appreciably affected by pasteurization and 16-18% decline in vitamin A concentration was observed after boiling and sterilisation. Kumar (1973) reported 8.4% loss of vitamin A in toned milk after pasteurisation. Maguer and Jackson (1983) assessed the content and stability of vitamin A in fortified milks and reported that destruction of 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. After pasteurization, vitamin A naturally present in milk (retinyl esters and alcohol) and synthetic form (retinyl palmitate) added during the fortification process showed non significant difference ($p>0.05$) in vitamin A stability.

### 4.11.2 Evaluation of the effect of light intensity on vitamin A stability in pasteurized milk

Control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) milk was pasteurized and packed in LDPE pouches and transparent glass bottles. These were then stored at refrigerated temperature (4-7°C) under three light intensities (1485, 2970 and 4455 lux). Vitamin A stability was analysed at a time interval of 12 h for the storage period of 48 h.

#### 4.11.2.1 Evaluation of the effect of light intensity on vitamin A stability in pasteurized milk packed in LDPE pouches

##### 4.11.2.1.1 Effect of light (1485 lux) on vitamin A stability of milk stored in LDPE pouches

Control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) pasteurised milk showed significant decrease ($p<0.05$) in vitamin A content (Table 4.52) after exposure to light (1485 lux) during storage (48 h).

**Table 4.52: Effect of light (1485 lux) on vitamin A content of milk stored in LDPE pouches**

| Storage period (h) | Vitamin A content (IU/L) | | | | |
|---|---|---|---|---|---|
| | Control (unfortified) | Vitamin A fortified milk | | | |
| | | Free vitamin A (oily form) | NaCas-VA ST | RNaCas-VA | RSNaCas-VA |
| **0** | 327.36±7.83$^{d}$ | 1810.32±24.32$^{d}$ | 1954.85±4.48$^{e}$ | 2005.08±29.34$^{d}$ | 2084.58±22.35$^{d}$ |
| **12** | 325.20±6.93$^{d}$ | 1555.73±23.42$^{d}$ | 1821.91±2.77$^{d}$ | 1974.95±25.08$^{d}$ | 2049.66±26.80$^{d}$ |
| **24** | 302.66±6.66$^{c}$ | 1311.22±13.87$^{c}$ | 1606.79±10.99$^{c}$ | 1772.69±21.54$^{c}$ | 1861.21±24.25$^{c}$ |
| **36** | 271.24±4.89$^{b}$ | 1177.84±18.32$^{b}$ | 1451.81±8.98$^{b}$ | 1618.59±13.31$^{b}$ | 1688.76±13.04$^{b}$ |
| **48** | 213.74±4.29$^{a}$ | 740.18±13.13$^{a}$ | 1071.07±6.31$^{a}$ | 1233.51±18.59$^{a}$ | 1306.58±19.04$^{a}$ |

Data are presented as means±SEM (n=3)
$^{a-b}$Means within rows with different superscript are significantly different ($p<0.05$) from each other.

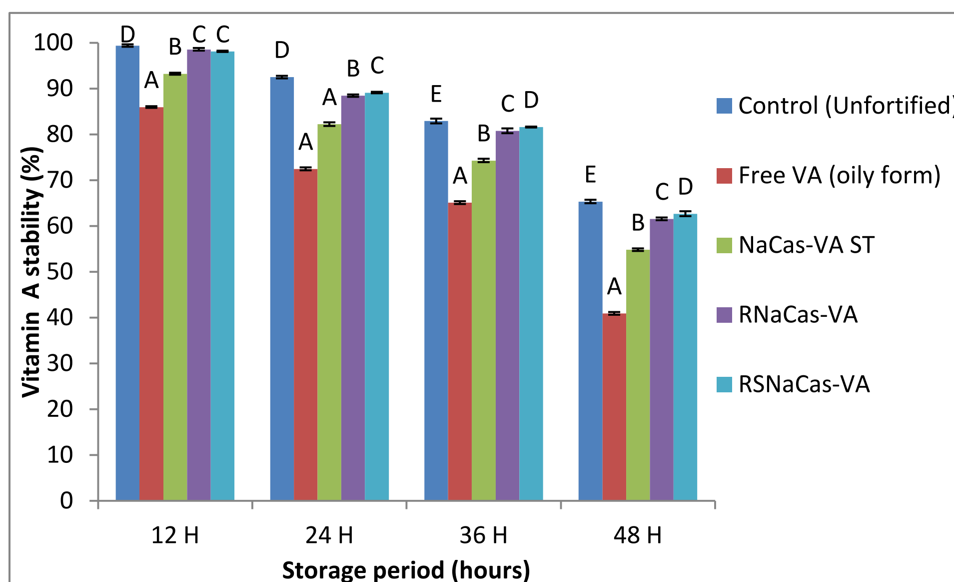
Table 4.53, figure 4.52 and 4.53 depict the stability of vitamin A in pasteurized milk stored in LDPE pouches exposed to light (1485 lux). Control (unfortified), free vitamin A (oily form), NaCas-VA ST, RNaCas-VA and RSNaCas-VA complex fortified milk showed 99.35, 85.93, 93.20, 98.53 and 98.09% vitamin A stability after

12 h of storage and 65.31, 40.88, 54.79, 61.52 and 62.65% vitamin A stability after 48 h of storage. Vitamin A stability in control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) milk was significantly different ($p<0.05$) from each other during the storage period of 48 h. Free vitamin A (oily form) fortified milk showed lowest vitamin A stability followed by NaCas-VA ST, RNaCas-VA, RSNaCas-VA complex fortified milk

**Table 4.53: Effect of light (1485 lux) on vitamin A stability of milk stored in LDPE pouches**

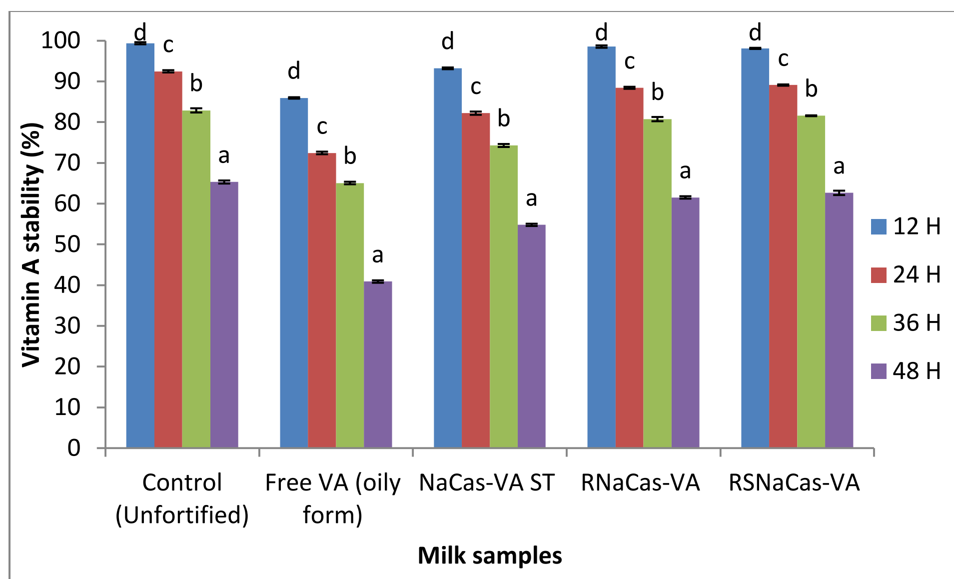
| Storage period (h) | Vitamin A stability (%) | | | | |
|---|---|---|---|---|---|
| | Control (unfortified) | Vitamin A fortified milk | | | |
| | | Free vitamin A (oily form) | NaCas-VA ST | RNaCas-VA | RSNaCas-VA |
| **12** | 99.35±0.26dD | 85.93±0.18dA | 93.20±0.22dB | 98.53±0.29dC | 98.09±0.14dC |
| **24** | 92.46±0.28cD | 82.44±0.32cA | 82.19±0.40cA | 88.42±0.25cB | 89.09±0.16cC |
| **36** | 82.88±0.52bE | 65.06±0.31bA | 74.27±0.37bB | 80.74±0.52bC | 81.56±0.10bD |
| **48** | 65.31±0.38aE | 40.88±0.30aA | 54.79±0.28aB | 61.52±0.30aC | 62.65±0.54aD |

Data are presented as means±SEM (n=3)
$^{a-b}$Means within rows with different superscript are significantly different ($p<0.05$) from each other.
$^{A-B}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.




$^{A-B}$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.

**Fig 4.52: Effect of light (1485 lux) on vitamin A stability in control and vitamin A fortified milk samples stored in LDPE pouches**




[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.

**Fig 4.53: Effect of light (1485 lux) on vitamin A stability in milk stored in LDPE pouches**

and control (unfortified) milk. RSNaCas-VA and RNaCas-VA complex fortified milk showed higher vitamin A stability as compared to NaCas-VA ST (Fig 4.52). Binding of free vitamin A (oily form) to milk protein improved the vitamin A stability. Control (unfortified) and vitamin A fortified milk samples showed significant decrease ($p<0.05$) in vitamin A stability during the storage period of 48 h (Fig 4.53).

#### 4.11.2.1.2 Effect of light (2970 lux) on vitamin A stability of milk stored in LDPE pouches

Control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) pasteurised milk sample showed significant decrease ($p<0.05$) in vitamin A content during exposure to light (2970 lux) for a storage period of 48 h (Table 4.54).

Table 4.55, figure 4.54 and 4.55 depict the stability of vitamin A in pasteurized milk stored in LDPE pouches exposed to light (2970 lux). Control (unfortified), free vitamin A (oily form), NaCas-VA ST, RNaCas-VA and RSNaCas-VA complex fortified milk showed 95.31, 76.39, 86.01, 92.41 and 94.10% vitamin A stability after 12 h of storage and 53.33, 35.04, 43.23, 49.40 and 50.80% vitamin A stability after

**Table 4.54: Effect of light (2970 lux) on vitamin A content of milk stored in LDPE pouches**

| Storage period (h) | Vitamin A content (IU/L) | | | | |
|---|---|---|---|---|---|
| | Control (unfortified) | Vitamin A fortified milk | | | |
| | | Free vitamin A (oily form) | NaCas-VA ST | RNaCas-VA | RSNaCas-VA |
| **0** | $361.46\pm8.06^{d}$ | $1963.73\pm29.51^{e}$ | $2170.43\pm10.00^{e}$ | $2207.11\pm17.68^{e}$ | $2302.43\pm27.65^{e}$ |
| **12** | $344.50\pm7.87^{d}$ | $1499.95\pm20.48^{d}$ | $1866.70\pm1.23^{d}$ | $2039.52\pm11.97^{d}$ | $2166.76\pm33.99^{d}$ |
| **24** | $297.64\pm5.64^{c}$ | $1239.33\pm19.89^{c}$ | $1539.23\pm4.11^{c}$ | $15655.73\pm18.90^{c}$ | $1800.77\pm14.61^{c}$ |
| **36** | $242.44\pm4.21^{b}$ | $943.19\pm14.23^{b}$ | $1215.07\pm3.38^{b}$ | $1371.85\pm16.34^{b}$ | $1429.65\pm26.55^{b}$ |
| **48** | $192.81\pm5.16^{a}$ | $687.82\pm3.65^{a}$ | $938.30\pm8.31^{a}$ | $1090.15\pm3.08^{a}$ | $1169.68\pm19.13^{a}$ |

Data are presented as means±SEM (n=3)

$^{a-b}$Means within rows with different superscript are significantly different (p<0.05) from each other.

48 h of storage. Vitamin A stability in control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) milk was significantly different (p<0.05) from each other during the storage period of 48 h. At 2970 lux, vitamin A stability in control (unfortified) and vitamin A fortified milk followed similar trend as reported at 1485 lux (Fig 4.54). Control (unfortified) and vitamin A fortified milk showed significant decrease (p<0.05) in vitamin A stability during the storage period of 48 h (Fig 4.55).

**Table 4.55: Effect of light (2970 lux) on vitamin A stability of milk stored in LDPE pouches**

| Storage period (h) | Vitamin A stability (%) | | | | |
|---|---|---|---|---|---|
| | Control (unfortified) | Vitamin A fortified milk | | | |
| | | Free vitamin A (oily form) | NaCas-VA ST | RNaCas-VA | RSNaCas-VA |
| **12** | $95.31\pm0.47^{dE}$ | $76.39\pm0.24^{dA}$ | $86.01\pm0.43^{dB}$ | $92.41\pm0.20^{dC}$ | $94.10\pm0.43^{dD}$ |
| **24** | $82.36\pm0.41^{cE}$ | $63.11\pm0.28^{cA}$ | $70.92\pm0.25^{cB}$ | $77.43\pm0.34^{cC}$ | $78.22\pm0.40^{cD}$ |
| **36** | $67.09\pm0.36^{bD}$ | $48.03\pm0.36^{bA}$ | $55.99\pm0.44^{bB}$ | $62.15\pm0.39^{bC}$ | $62.08\pm0.42^{bC}$ |
| **48** | $53.33\pm0.25^{aE}$ | $35.04\pm0.35^{aA}$ | $43.23\pm0.26^{aB}$ | $49.40\pm0.27^{aC}$ | $50.80\pm0.23^{aD}$ |

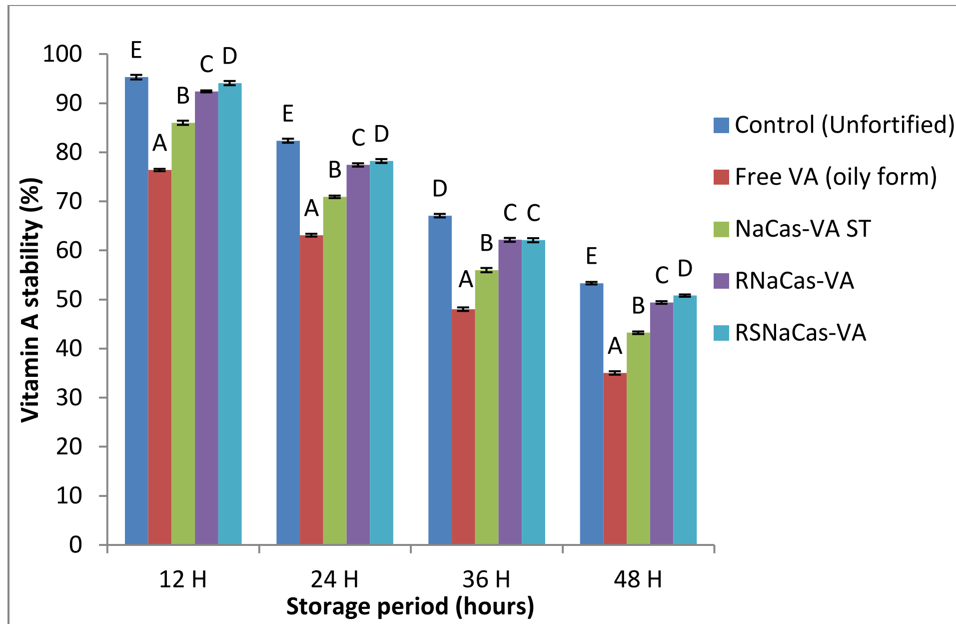
Data are presented as means±SEM (n=3)

$^{a-b}$Means within rows with different superscript are significantly different (p<0.05) from each other.

$^{A-B}$Means within columns with different superscript are significantly different (p<0.05) from each other.

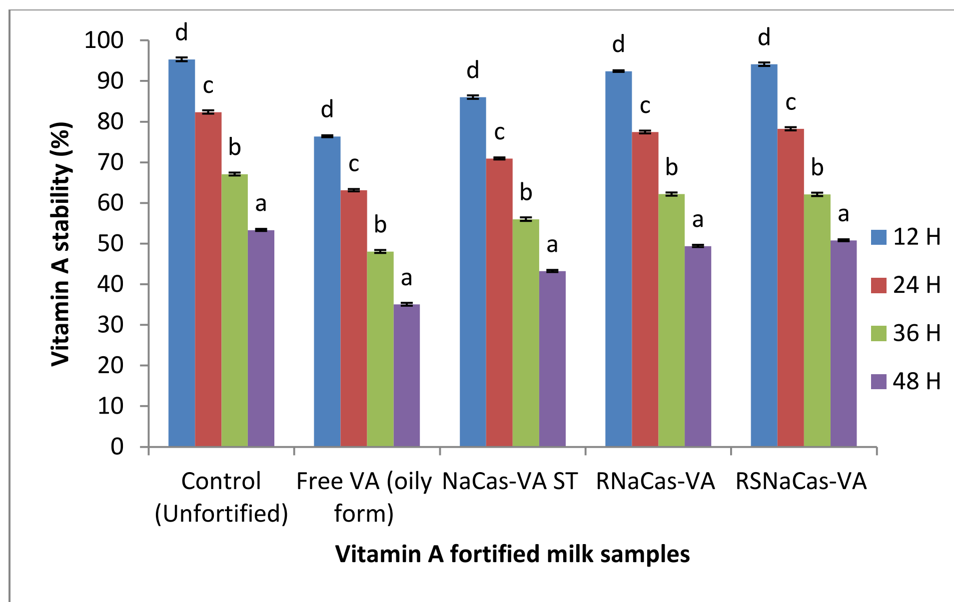
#### 4.11.2.1.3 Effect of light (4455 lux) on vitamin A stability of milk stored in LDPE pouches

Control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) pasteurised milk samples




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.

**Fig 4.54: Effect of light (2970 lux) on vitamin A stability in control (unfortified) and vitamin A fortified milk samples stored in LDPE pouches**




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.

**Fig 4.55: Effect of light (2970 lux) on vitamin A stability in milk stored in LDPE pouches**

showed significant decrease (p<0.05) in vitamin A content during exposure to light (4455 lux) for a storage period of 48 h (Table 4.56).

**Table 4.56: Effect of light (4455 lux) on vitamin A content of milk stored in LDPE pouches**

| Storage period (h) | Vitamin A content (IU/L) | | | | |
|---|---|---|---|---|---|
| | Control (unfortified) | Vitamin A fortified milk | | | |
| | | Free vitamin A (oily form) | NaCas-VA ST | RNaCas-VA | RSNaCas-VA |
| **0** | 394.18±7.70$^{e}$ | 2421.51±29.59$^{e}$ | 2413.75±11.49$^{e}$ | 2393.47±22.68$^{e}$ | 2455.40±32.01$^{e}$ |
| **12** | 346.60±7.24$^{d}$ | 1674.94±27.07$^{d}$ | 1894.33±13.22$^{d}$ | 2029.79±23.14$^{d}$ | 2109.64±35.53$^{d}$ |
| **24** | 244.78±4.85$^{c}$ | 1043.42±3.08$^{c}$ | 1266.88±10.02$^{c}$ | 1407.24±3.09$^{c}$ | 1493.34±20.62$^{c}$ |
| **36** | 217.66±2.56$^{b}$ | 862.56±8.18$^{b}$ | 1093.57±6.40$^{b}$ | 1229.31±7.17$^{b}$ | 1276.14±20.08$^{b}$ |
| **48** | 178.77±3.14$^{a}$ | 675.60±7.92$^{a}$ | 864.98±5.37$^{a}$ | 999.99±22.95$^{a}$ | 1028.61±9.23$^{a}$ |

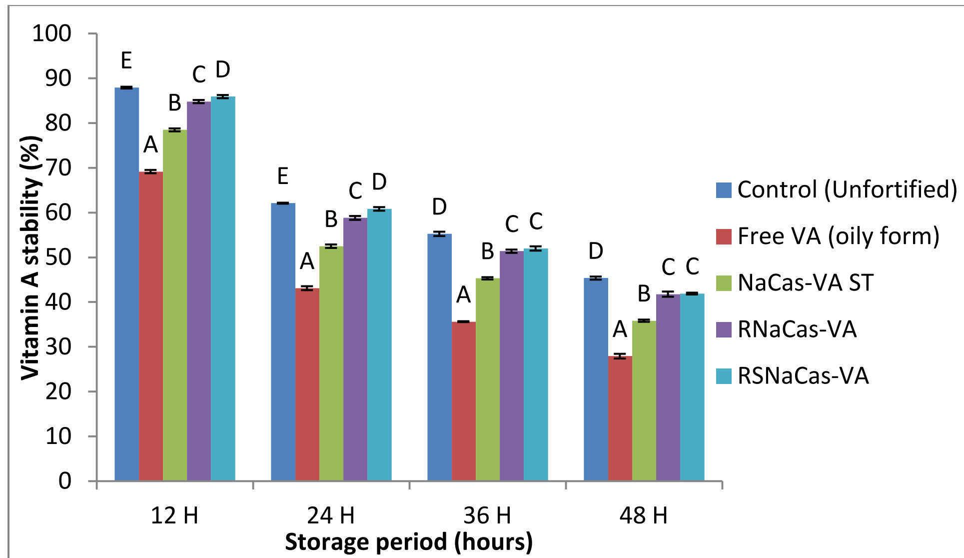
Data are presented as means±SEM (n=3)
$^{a-b}$Means within rows with different superscript are significantly different (p<0.05) from each other.

Table 4.57, figure 4.56 and 4.57 depict the stability of vitamin A in pasteurized milk stored in LDPE pouches exposed to light (4455 lux). Control (unfortified), free vitamin A (oily form), NaCas-VA ST, RNaCas-VA and RSNaCas-VA complex fortified milk showed 87.92, 69.16, 78.48, 84.80 and 85.91% vitamin A stability after 12 h of storage and 45.36, 27.91, 35.84, 41.77 and 41.90% vitamin A stability after 48 h of storage. Vitamin A stability in control (unfortified) and vitamin A fortified

**Table 4.57: Effect of light (4455 lux) on vitamin A stability of milk stored in LDPE pouches**

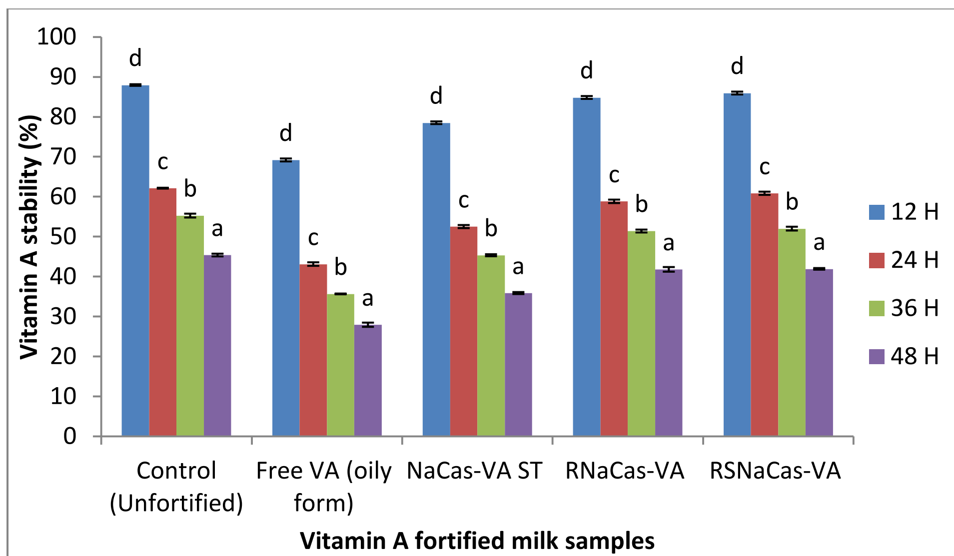
| Storage period (h) | Vitamin A stability (%) | | | | |
|---|---|---|---|---|---|
| | Control (unfortified) | Vitamin A fortified milk | | | |
| | | Free vitamin A (oily form) | NaCas-VA ST | RNaCas-VA | RSNaCas-VA |
| **12** | 87.92±0.20$^{dE}$ | 69.16±0.37$^{dA}$ | 78.48±0.33$^{dB}$ | 84.80±0.35$^{dC}$ | 85.91±0.36$^{dD}$ |
| **24** | 62.10±0.11$^{cE}$ | 43.10±0.44$^{cA}$ | 52.49±0.39$^{cB}$ | 58.80±0.43$^{cC}$ | 60.82±0.40$^{cD}$ |
| **36** | 55.24±0.48$^{bD}$ | 35.62±0.10$^{bA}$ | 45.31±0.25$^{bB}$ | 51.37±0.36$^{bC}$ | 51.97±0.48$^{bC}$ |
| **48** | 45.36±0.34$^{aD}$ | 27.91±0.52$^{aA}$ | 35.84±0.25$^{aB}$ | 41.77±0.58$^{aC}$ | 41.90±0.20$^{aC}$ |

Data are presented as means±SEM (n=3)
$^{a-b}$Means within rows with different superscript are significantly different (p<0.05) from each other.
$^{A-B}$Means within columns with different superscript are significantly different (p<0.05) from each other.




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.

**Fig 4.56: Effect of light (4455 lux) on vitamin A stability in control and vitamin A fortified milk samples stored in LDPE pouches**




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.

**Fig 4.57: Effect of light (4455 lux) on vitamin A stability in milk stored in LDPE pouches**

(containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) milk was significantly different ($p<0.05$) from each other during the storage period of 48 h. At 4455 lux, vitamin A stability in control (unfortified) and vitamin A fortified milk followed similar trend as reported at 1485 and 2970 lux (Fig 4.56).

Control (unfortified) and vitamin A fortified milk showed significant decrease ($p<0.05$) in vitamin A stability during the storage period of 48 h (Fig 4.57).

Control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) pasteurised milk showed lowest vitamin A stability during exposure to light (4455 lux followed by exposure to 2970 lux and 1485 lux) when packed in LDPE pouches.

### 4.11.2.2 Evaluation of the effect of light intensity on vitamin A stability in pasteurized milk packed in transparent glass bottles

#### 4.11.2.2.1 Effect of light (1485 lux) on vitamin A stability of milk stored in glass bottles

Control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) pasteurised milk sample showed significant decrease ($p<0.05$) in vitamin A content during exposure to light (1485 lux) for a storage period of 48 h (Table 4.58).

**Table 4.58: Effect of light (1485 lux) on vitamin A content of milk stored in glass bottles**

| Storage period (h) | Vitamin A content (IU/L) | | | | |
|---|---|---|---|---|---|
| | Control (unfortified) | Vitamin A fortified milk | | | |
| | | Free vitamin A (oily form) | NaCas-VA ST | RNaCas-VA | RSNaCas-VA |
| **0** | 347.68±8.48$^{d}$ | 1925.06±29.56$^{e}$ | 1984.36±19.29$^{e}$ | 2015.10±20.02$^{e}$ | 2080.81±40.76$^{d}$ |
| **12** | 333.77±8.53$^{d}$ | 1492.39±22.21$^{d}$ | 1722.55±9.98$^{d}$ | 1901.32±16.69$^{d}$ | 1981.80±36.77$^{d}$ |
| **24** | 301.84±7.47$^{c}$ | 1326.16±20.35$^{c}$ | 1556.26±10.64$^{c}$ | 1712.77±23.68$^{c}$ | 1786.69±39.69$^{c}$ |
| **36** | 250.93±6.73$^{b}$ | 984.89±22.26$^{b}$ | 1235.40±6.27$^{b}$ | 1396.33±14.78$^{b}$ | 1493.79±27.30$^{b}$ |
| **48** | 203.20±5.14$^{a}$ | 719.26±19.87$^{a}$ | 988.67±7.27$^{a}$ | 1147.32±11.38$^{a}$ | 1205.10±30.18$^{a}$ |

Data are presented as means±SEM (n=3)
$^{a\text{-}b}$Means within rows with different superscript are significantly different ($p<0.05$) from each other.

Table 4.59, figure 4.58 and 4.59 depict the stability of vitamin A in pasteurized milk stored in glass bottles exposed to light (1485 lux). Control (unfortified), free vitamin A (oily form), NaCas-VA ST, RNaCas-VA and RSNaCas-VA complex fortified milk showed 95.99, 77.53, 86.81, 94.36 and 95.25% vitamin A stability after 12 h of storage and 58.44, 37.37, 49.83, 56.94 and 57.90% vitamin A stability after 48 h of storage. Vitamin A stability in control (unfortified) and vitamin A fortified

(containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) milk was significantly different ($p<0.05$) from each other during the storage period of 48 h. Free vitamin A (oily form) fortified milk showed lowest vitamin A stability followed by NaCas-VA ST, RNaCas-VA, RSNaCas-VA complex and control (unfortified) milk. RSNaCas-VA and RNaCas-VA complex fortified milk showed higher vitamin A stability as compared to NaCas-VA ST (Fig 4.58). Binding of free

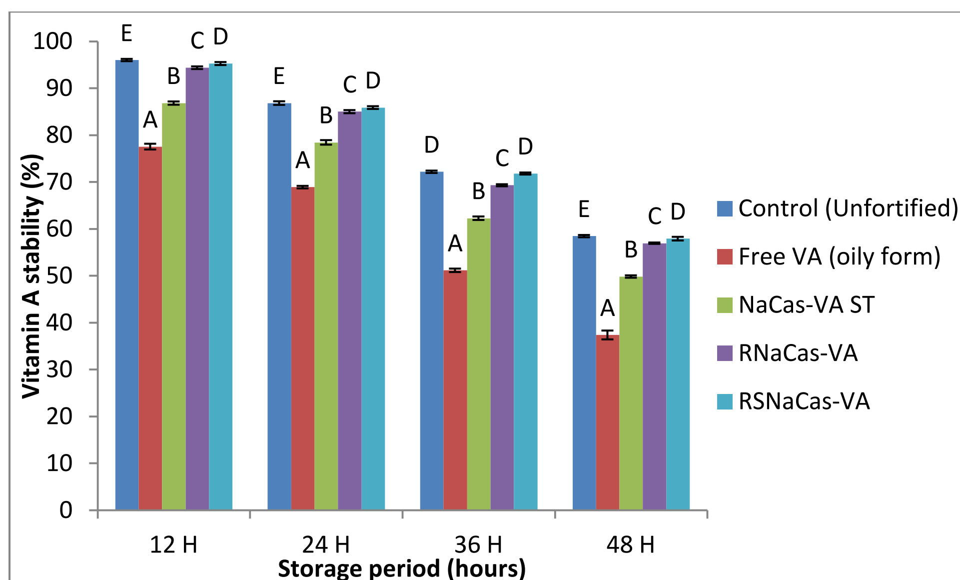
**Table 4.59: Effect of light (1485 lux) on vitamin A stability of milk stored in glass bottles**

| Storage period (h) | Vitamin A stability (%) | | | | |
|---|---|---|---|---|---|
| | Control (unfortified) | Vitamin A fortified milk | | | |
| | | Free vitamin A (oily form) | NaCas-VA ST | RNaCas-VA | RSNaCas-VA |
| **12** | $95.99 \pm 0.24^{dE}$ | $77.53 \pm 0.61^{dA}$ | $86.81 \pm 0.35^{dB}$ | $94.36 \pm 0.29^{dC}$ | $95.25 \pm 0.32^{Dd}$ |
| **24** | $86.81 \pm 0.39^{cE}$ | $68.89 \pm 0.27^{cA}$ | $78.43 \pm 0.48^{cB}$ | $84.99 \pm 0.33^{cC}$ | $85.86 \pm 0.29^{cD}$ |
| **36** | $72.16 \pm 0.25^{bD}$ | $51.15 \pm 0.37^{bA}$ | $62.26 \pm 0.37^{bB}$ | $69.29 \pm 0.22^{bC}$ | $71.79 \pm 0.22^{Bd}$ |
| **48** | $58.44 \pm 0.24^{aE}$ | $37.37 \pm 0.94^{aA}$ | $49.83 \pm 0.25^{aB}$ | $56.94 \pm 0.15^{aC}$ | $57.90 \pm 0.39^{Ad}$ |

Data are presented as means±SEM (n=3)

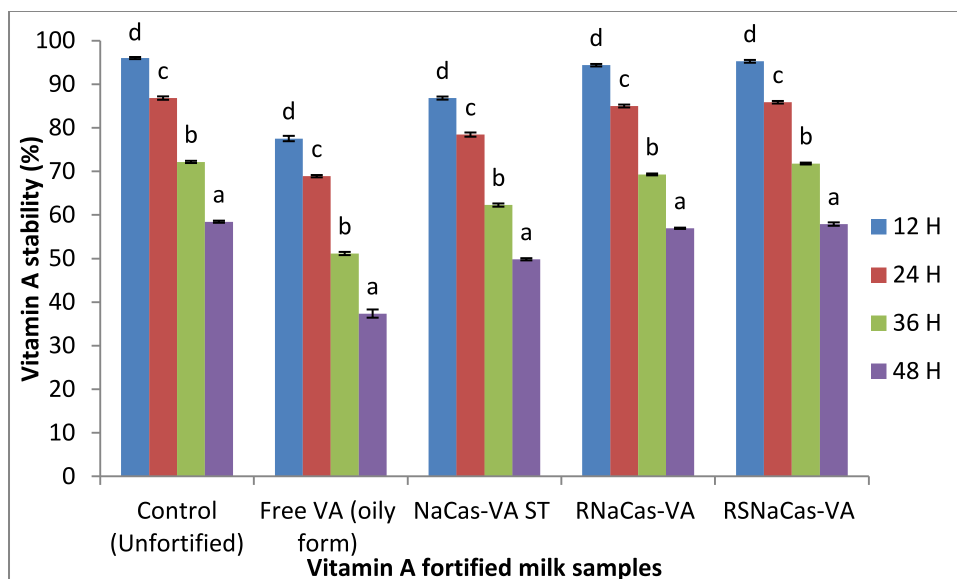
$^{a-b}$Means within rows with different superscript are significantly different ($p<0.05$) from each other.

$^{A-B}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.




$^{A-B}$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.

**Fig 4.58: Effect of light (1485 lux) on vitamin A stability in control (unfortified) and vitamin A fortified milk samples stored in glass bottles**




[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.

**Fig 4.59: Effect of light (1485 lux) on vitamin A stability in milk stored in glass bottles**

vitamin A (oily form) to milk protein improved the vitamin A stability. Control (unfortified) and vitamin A fortified milk samples showed significant decrease ($p<0.05$) in vitamin A stability during the storage period of 48 h (Fig 4.59).

#### 4.11.2.2.2 Effect of light (2970 lux) on vitamin A stability of milk stored in glass bottles

Control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) pasteurised milk sample showed significant decrease ($p<0.05$) in vitamin A content during exposure to light (2970 lux) for a storage period of 48 h (Table 4.60).

Table 4.61, figure 4.60 and 4.61 depict the stability of vitamin A in pasteurized milk stored in glass bottles exposed to light (2970 lux). Control (unfortified), free vitamin A (oily form), NaCas-VA ST, RNaCas-VA and RSNaCas-VA complex fortified milk showed 90.69, 72.84, 82.41, 89.86 and 90.38% vitamin A stability after 12 h of storage and 47.88, 30.95, 40.81, 46.88 and 46.92% vitamin A stability after 48 h of storage. Vitamin A stability in control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily

**Table 4.60: Effect of light (2970 lux) on vitamin A content of milk stored in glass bottles**

| Storage period (h) | Vitamin A content (IU/L) | | | | |
|---|---|---|---|---|---|
| | Control (unfortified) | Vitamin A fortified milk | | | |
| | | Free vitamin A (oily form) | NaCas-VA ST | RNaCas-VA | RSNaCas-VA |
| **0** | 378.57±8.42$^{e}$ | 2140.51±22.72$^{e}$ | 2213.11±14.88$^{e}$ | 2277.33±26.95$^{e}$ | 2374.59±36.33$^{e}$ |
| **12** | 343.36±8.32$^{d}$ | 1559.12±19.05$^{d}$ | 1823.63±5.94$^{d}$ | 2046.44±29.00$^{d}$ | 2146.40±44.45$^{d}$ |
| **24** | 267.84±4.99$^{c}$ | 1054.62±17.72$^{c}$ | 1279.16±13.67$^{c}$ | 1488.96±22.75$^{c}$ | 1568.54±22.13$^{c}$ |
| **36** | 210.73±4.57$^{b}$ | 817.01±8.64$^{b}$ | 1023.12±9.82$^{b}$ | 1175.44±28.75$^{b}$ | 1235.61±26.54$^{b}$ |
| **48** | 181.23±3.36$^{a}$ | 662.71±15.05$^{a}$ | 903.16±2.05$^{a}$ | 1067.36±6.40$^{a}$ | 1114.39±23.42$^{a}$ |

Data are presented as means±SEM (n=3)
$^{a-b}$Means within rows with different superscript are significantly different (p<0.05) from each other.

form)) milk was significantly different (p<0.05) from each other during the storage period of 48 h. At 2970 lux, vitamin A stability in control (unfortified) and vitamin A fortified milk followed similar trend as reported at 1485 lux (Fig 4.60). Control (unfortified) and vitamin A fortified milk showed significant decrease (p<0.05) in vitamin A stability during the storage period of 48 h (Fig 4.61).

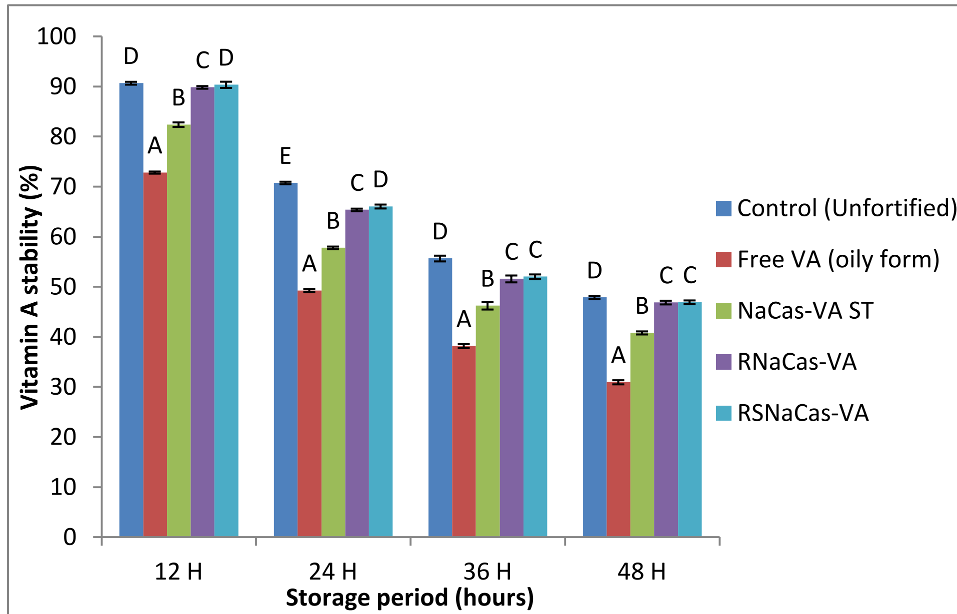
**Table 4.61: Effect of light (2970 lux) on vitamin A stability of milk stored in glass bottles**

| Storage period (h) | Vitamin A stability (%) | | | | |
|---|---|---|---|---|---|
| | Control (unfortified) | Vitamin A fortified milk | | | |
| | | Free vitamin A (oily form) | NaCas-VA ST | RNaCas-VA | RSNaCas-VA |
| **12** | 90.69±0.29$^{dD}$ | 72.84±0.21$^{dA}$ | 82.41±0.45$^{dB}$ | 89.86±0.26$^{dC}$ | 90.38±0.61$^{cCD}$ |
| **24** | 70.76±0.26$^{cE}$ | 49.26±0.31$^{cA}$ | 57.80±0.27$^{cB}$ | 65.38±0.26$^{cC}$ | 66.06±0.39$^{cD}$ |
| **36** | 55.67±0.58$^{bD}$ | 38.17±0.41$^{bA}$ | 46.24±0.75$^{bB}$ | 51.60±0.69$^{bC}$ | 52.03±0.47$^{bC}$ |
| **48** | 47.88±0.31$^{aD}$ | 30.95±0.41$^{aA}$ | 40.81±0.31$^{aB}$ | 46.88±0.37$^{aC}$ | 46.92±0.38$^{aC}$ |

Data are presented as means±SEM (n=3)
$^{a-b}$Means within rows with different superscript are significantly different (p<0.05) from each other.
$^{A-B}$Means within columns with different superscript are significantly different (p<0.05) from each other.

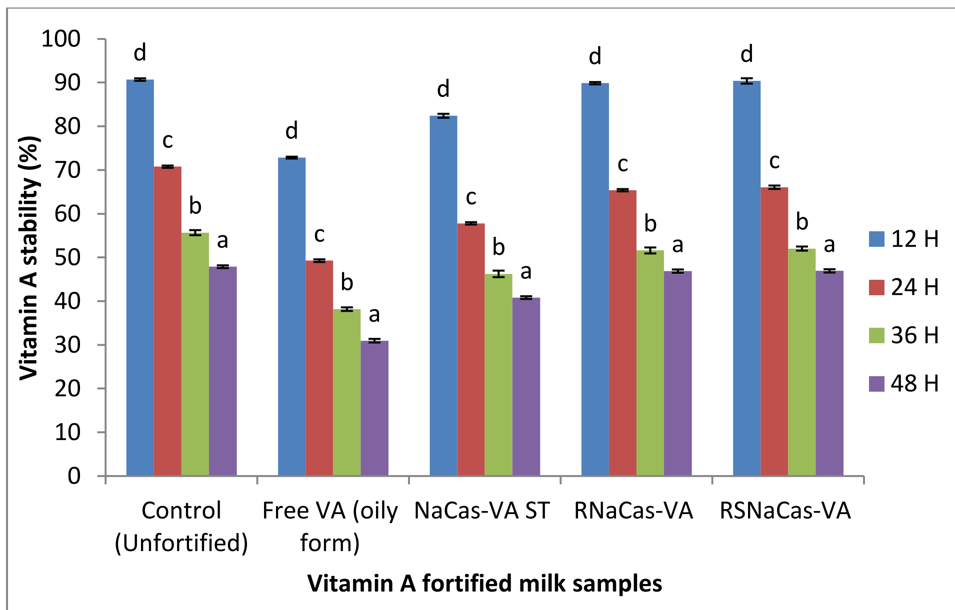
### 4.11.2.2.3 Effect of light (4455 lux) on vitamin A stability of milk stored in glass bottles

Control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) pasteurised milk sample




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.

**Fig 4.60: Effect of light (2970 lux) on vitamin A stability in control and vitamin A fortified milk samples stored in glass bottles**




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.

**Fig 4.61: Effect of light (2970 lux) on vitamin A stability in milk stored in glass bottles**

showed significant decrease ($p<0.05$) in vitamin A content during exposure to light (4455 lux) for a storage period of 48 h (Table 4.62).

**Table 4.62: Effect of light (4455 lux) on vitamin A content of milk stored in glass bottles**

| Storage period (h) | Vitamin A content (IU/L) | | | | |
|---|---|---|---|---|---|
| | Control (unfortified) | Vitamin A fortified milk | | | |
| | | Free vitamin A (oily form) | NaCas-VA ST | RNaCas-VA | RSNaCas-VA |
| **0** | 394.18±7.70$^{e}$ | 2421.51±29.59$^{e}$ | 2413.75±11.49$^{e}$ | 2393.47±22.68$^{e}$ | 2455.40±32.01$^{e}$ |
| **12** | 330.33±6.04$^{d}$ | 1588.87±14.10$^{d}$ | 1796.51±21.06$^{d}$ | 1945.22±18.13$^{d}$ | 2018.57±32.03$^{d}$ |
| **24** | 223.32±5.06$^{c}$ | 941.94±11.33$^{c}$ | 1172.19±6.81$^{c}$ | 1314.87±19.90$^{c}$ | 1369.90±14.13$^{c}$ |
| **36** | 184.87±3.89$^{b}$ | 654.97±4.45$^{b}$ | 871.89±2.16$^{b}$ | 1028.82±5.36$^{b}$ | 1075.25±12.99$^{b}$ |
| **48** | 150.26±2.12$^{a}$ | 525.27±13.44$^{a}$ | 744.02±6.70$^{a}$ | 854.87±5.04$^{a}$ | 907.58±17.59$^{a}$ |

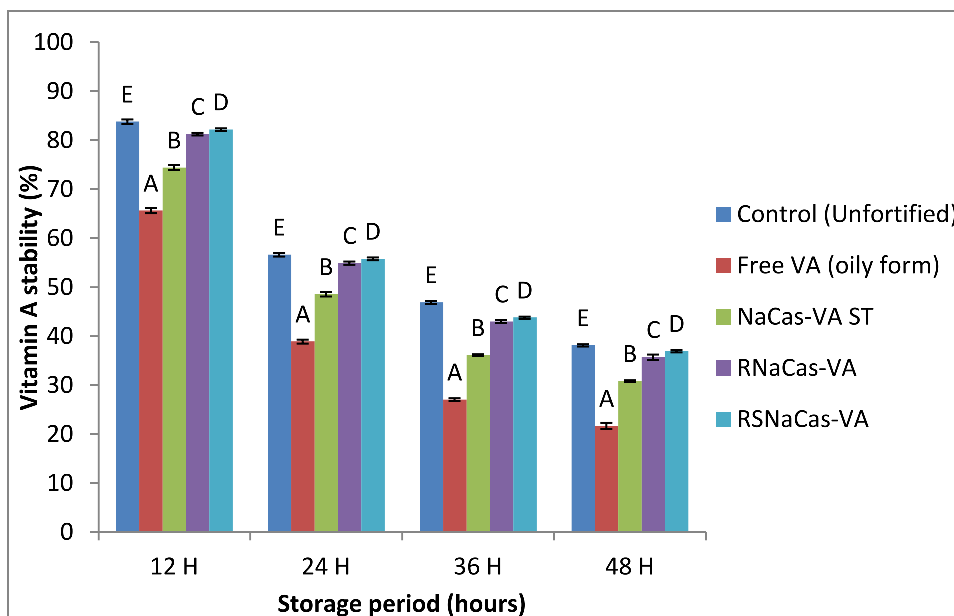
Data are presented as means±SEM (n=3)
$^{a-b}$Means within rows with different superscript are significantly different ($p<0.05$) from each other.

Table 4.63, figure 4.62 and 4.63 depict the stability of vitamin A in pasteurized milk stored in glass bottles exposed to light (4455 lux). Control (unfortified), free vitamin A (oily form), NaCas-VA ST, RNaCas-VA and RSNaCas-VA complex fortified milk showed 83.81, 65.62, 74.42, 81.27 and 82.20% vitamin A stability after 12 h of storage and 38.13, 21.70, 30.82, 35.73 and 36.96% vitamin A stability after 48 h of storage. Vitamin A stability in control (unfortified) and vitamin A fortified

**Table 4.63: Effect of light (4455 lux) on vitamin A stability of milk stored in glass bottles**

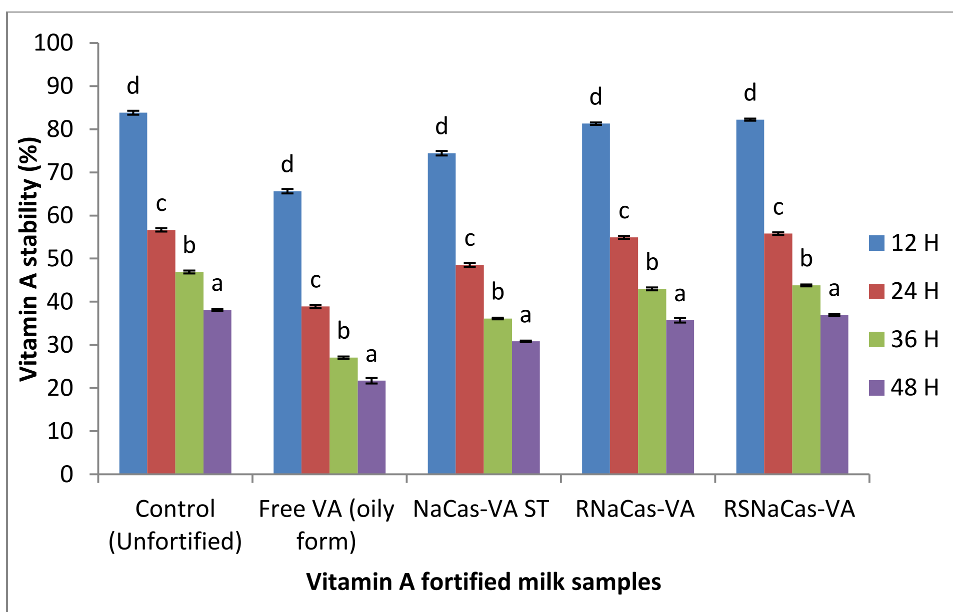
| Storage period (h) | Vitamin A stability (%) | | | | |
|---|---|---|---|---|---|
| | Control (unfortified) | Vitamin A fortified milk | | | |
| | | Free vitamin A (oily form) | NaCas-VA ST | RNaCas-VA | RSNaCas-VA |
| **12** | 83.81±0.46dE | 65.62±0.51dA | 74.42±0.53dB | 81.27±0.28dC | 82.20±0.25dD |
| **24** | 56.65±0.38cE | 38.90±0.41cA | 48.57±0.45cB | 54.93±0.33cC | 55.80±0.29cD |
| **36** | 46.90±0.34bE | 27.05±0.27bA | 36.12±0.19bB | 42.99±0.34bC | 43.79±0.22bD |
| **48** | 38.13±0.22aE | 21.70±0.64aA | 30.82±0.18aB | 35.73±0.54aC | 36.96±0.26aD |

Data are presented as means±SEM (n=3)
$^{a-b}$Means within rows with different superscript are significantly different ($p<0.05$) from each other.
$^{A-B}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.




[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.

**Fig 4.62: Effect of light (4455 lux) on vitamin A stability in control (unfortified) and vitamin A fortified milk samples stored in glass bottles**




[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.

**Fig 4.63: Effect of light (4455 lux) on vitamin A stability in milk stored in glass bottles**

(containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) milk was significantly different ($p<0.05$) from each other during the storage period of 48 h. At 4455 lux, vitamin A stability in control (unfortified) and vitamin A fortified milk followed similar trend as reported at 1485 and 2970 lux (Fig 4.62). Control (unfortified) and vitamin A fortified milk showed significant decrease ($p<0.05$) in vitamin A stability during the storage period of 48 h (Fig 4.63).

Control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) pasteurised milk packed in glass bottles showed lowest vitamin A stability when exposed to light (4455 lux followed by 2970 lux and 1485 lux). Our results are in accordance with Gaylord *et al.* (1986) who reported that the loss in retinyl palmitate in fortified milk increased with the increase in light intensity and duration of storage when packed in glass bottles. Thompson and Erdody (1974) reported that vitamin A added to milk is much more sensitive to light than native vitamin A. Exposure of milk to fluorescent light resulted in 50% loss of added vitamin A after 3 h of exposure and no loss of native vitamin A even after 5 h of exposure. Bartholomew and Ogden (1990) reported the vitamin A content decreased during storage period of 32 h after exposure of fortified milk to light intensity 4300 lux. Zahar *et al.* (1987) reported light induced isomerisation as most probable mechanism for vitamin A degradation rather than autoxidation. Senyk and Shipe (1981) reported reduction in the vitamin A content of whole milk, reduced fat, low fat and non fat milk exposed to light intensity 2000 lux for 4 h. Whited *et al*. (2002) reported that measurable losses of vitamin A occurred after exposure of non fat, reduced fat and whole milk to light intensity 2000 lux for 2, 4 and 16 h. deMan (1981) reported the loss of vitamin A in whole, 2% and skim milk after exposure to 2200 lux for 48 h and found highest losses (95.8%) in skim milk. 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%, which increased with the period of exposure. 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 in 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 the losses 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.

Control (unfortified) and vitamin A fortified milk samples showed higher stability of vitamin A in LDPE pouches in comparison to glass bottles after exposure to light (1485, 2970 and 4455 lux). Our results are in accordance with Saffert *et al.* (2008) who reported higher losses of vitamin A in clear polyethylene terephthalate (PET) bottles as compared to pigmented PET bottles. Storage of milk in dark further reduces the vitamin A losses. Cladman *et al.* (1998) reported that vitamin A stability was lowest in HDPE pouches followed by clear PET bottles, LDPE pouches and green PET bottles (containing UV blockers). It was clearly evident that blocking of visible light with translucent labels can help in reducing vitamin A degradation.

#### 4.11.3 Evaluation of the effect of packaging material on vitamin A stability

Control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) pasteurised milk were stored at refrigerated temperature (4-7°C) using two different packaging materials i.e. LDPE pouches and glass bottles. Vitamin A content was estimated on 0, 3$^{rd}$, 5$^{th}$ and 7$^{th}$ day of storage.

##### 4.11.3.1 Effect of storage in LDPE pouches on stability of vitamin A in milk

Control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) pasteurised milk samples showed significant decrease ($p<0.05$) in vitamin A content during storage in LDPE pouches for a period of 7 days (Table 4.64).

Table 4.65, figure 4.64 and 4.65 represent the vitamin A stability of milk stored in LDPE pouches at 4-7ºC. Control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) milk samples showed significant decrease ($p<0.05$) in vitamin A stability of milk stored in LDPE pouches on 3$^{rd}$, 5$^{th}$ and 7$^{th}$ day of storage (Fig 4.64). On 3$^{rd}$ day of storage, free vitamin A (oily form) fortified milk showed lowest vitamin A stability followed by NaCas-VA ST, control (unfortified), RNaCas-VA and RSNaCas-VA.

**Table 4.64: Effect of storage (4-7ºC) in LDPE pouches on vitamin A content of milk**

| Storage period (day) | Vitamin A content (IU/L) | | | | |
|---|---|---|---|---|---|
| | Control (unfortified) | Vitamin A fortified milk | | | |
| | | Free vitamin A (oily form) | NaCas-VA ST | RNaCas-VA | RSNaCas-VA |
| **0** | $394.18 \pm 7.70^{d}$ | $2421.51 \pm 29.59^{d}$ | $2413.75 \pm 11.49^{d}$ | $2393.47 \pm 22.68^{d}$ | $2455.40 \pm 32.01^{d}$ |
| **3** | $361.46 \pm 8.06^{c}$ | $1963.73 \pm 29.51^{c}$ | $2170.43 \pm 10.01^{c}$ | $2207.11 \pm 17.68^{c}$ | $2302.43 \pm 27.65^{c}$ |
| **5** | $327.36 \pm 7.83^{b}$ | $1810.32 \pm 24.32^{b}$ | $1954.85 \pm 4.48^{b}$ | $2005.08 \pm 29.34^{b}$ | $2084.58 \pm 22.35^{b}$ |
| **7** | $270.77 \pm 5.57^{a}$ | $1207.55 \pm 10.14^{a}$ | $1503.28 \pm 4.57^{a}$ | $1580.33 \pm 16.21^{a}$ | $1638.63 \pm 20.82^{a}$ |

Data are presented as means±SEM (n=3)
$^{a-b}$Means within rows with different superscript are significantly different (p<0.05) from each other.

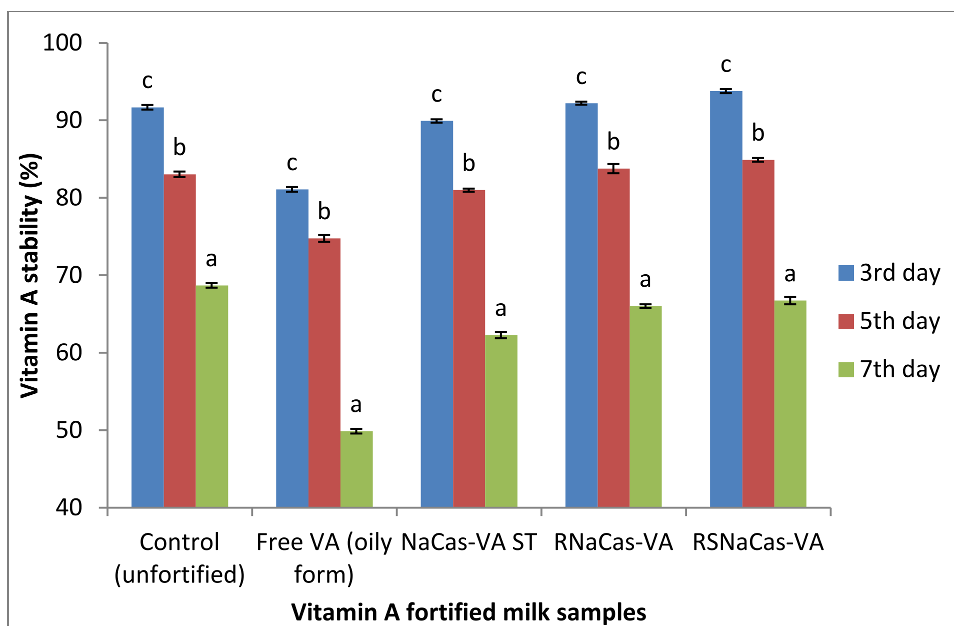
Control (unfortified) and RNaCas-VA complex fortified milk showed non significant difference (p>0.05) in vitamin A stability. However, RSNaCas-VA complex fortified milk showed slight but significant difference (p<0.05) in vitamin A stability as compared to control (unfortified) and RNaCas-VA complex fortified milk. On 5$^{th}$ day of storage, free vitamin A (oily form) fortified milk showed lowest vitamin A stability followed by NaCas-VA ST, control (unfortified), RNaCas-VA and RSNaCas-VA. Control (unfortified), RNaCas-VA and RSNaCas-VA complex fortified milk showed slight but significant difference (p<0.05) in vitamin A stability from each other.

**Table 4.65: Effect of storage (4-7ºC) in LDPE pouches on vitamin A stability of milk**

| Storage period (day) | Vitamin A stability (%) | | | | |
|---|---|---|---|---|---|
| | Control (unfortified) | Vitamin A fortified milk | | | |
| | | Free vitamin A (oily form) | NaCas-VA ST | RNaCas-VA | RSNaCas-VA |
| **3** | $91.69 \pm 0.29^{cC}$ | $81.09 \pm 0.29^{cA}$ | $89.92 \pm 0.22^{cB}$ | $92.22 \pm 0.19^{cC}$ | $93.77 \pm 0.26^{cD}$ |
| **5** | $83.03 \pm 0.36^{bC}$ | $74.76 \pm 0.43^{bA}$ | $80.99 \pm 0.20^{bB}$ | $83.77 \pm 0.60^{bD}$ | $84.90 \pm 0.24^{bE}$ |
| **7** | $68.69 \pm 0.29^{aD}$ | $49.87 \pm 0.30^{aA}$ | $62.28 \pm 0.42^{aB}$ | $66.03 \pm 0.23^{aC}$ | $66.74 \pm 0.49^{aC}$ |

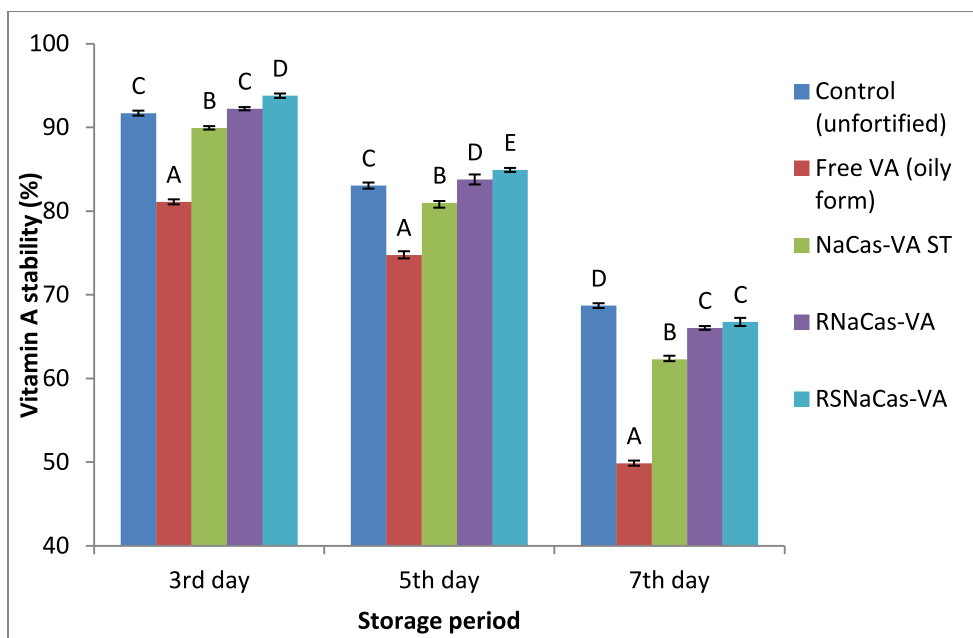
Data are presented as means±SEM (n=3)
$^{a-b}$Means within columns with different superscript are significantly different (p<0.05) from each other.
$^{A-B}$Means within rows with different superscript are significantly different (p<0.05) from each other.

On 7$^{th}$ day of storage, free vitamin A (oily form) fortified milk showed lowest vitamin A stability followed by NaCas-VA ST, RNaCas-VA, RSNaCas-VA and control (unfortified) milk. RNaCas-VA and RSNaCas-VA complexes fortified milk showed non significant difference (p>0.05) in vitamin A stability. However, control




[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.

**Fig 4.64: Effect of storage (4-7°C) in LDPE pouches on vitamin A stability of milk**




[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.

**Fig 4.65: Comparison of vitamin A stability in control and vitamin A fortified milk samples as affected by storage (4-7$^{o}$C) in LDPE pouches**

(unfortified) milk showed slight but significant difference ($p<0.05$) in vitamin A stability as compared to RNaCas-VA and RSNaCas-VA complexes fortified milk (Fig 4.65).

#### 4.11.3.2 Effect of storage in transparent glass bottles on stability of vitamin A in milk

Control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) pasteurised milk samples showed significant decrease ($p<0.05$) in vitamin A content during storage in glass bottles for a period of 7 days (Table 4.66).

**Table 4.66: Effect of storage (4-7ºC) in glass bottles on vitamin A content of milk**

| Storage period (day) | Vitamin A content (IU/L) | | | | |
|---|---|---|---|---|---|
| | Control (unfortified) | Vitamin A fortified milk | | | |
| | | Free vitamin A (oily form) | NaCas-VA ST | RNaCas-VA | RSNaCas-VA |
| 0 | 394.18±7.70$^{d}$ | 2421.51±29.59$^{d}$ | 2413.75±11.49$^{d}$ | 2393.47±22.67$^{d}$ | 2455.40±32.00$^{d}$ |
| 3 | 378.57±8.42$^{c}$ | 2140.51±22.72$^{c}$ | 2213.11±14.88$^{c}$ | 2277.33±26.95$^{c}$ | 2374.59±36.33$^{c}$ |
| 5 | 347.68±8.48$^{b}$ | 1925.06±29.56$^{b}$ | 1984.36±19.29$^{b}$ | 2054.90±21.37$^{b}$ | 2129.92±41.39$^{b}$ |
| 7 | 283.74±4.82$^{a}$ | 1438.41±27.80$^{a}$ | 1552.36±1.77$^{a}$ | 1633.17±25.42$^{a}$ | 1700.20±32.64$^{a}$ |

Data are presented as means±SEM (n=3)
$^{a-b}$Means within rows with different superscript are significantly different ($p<0.05$) from each other.

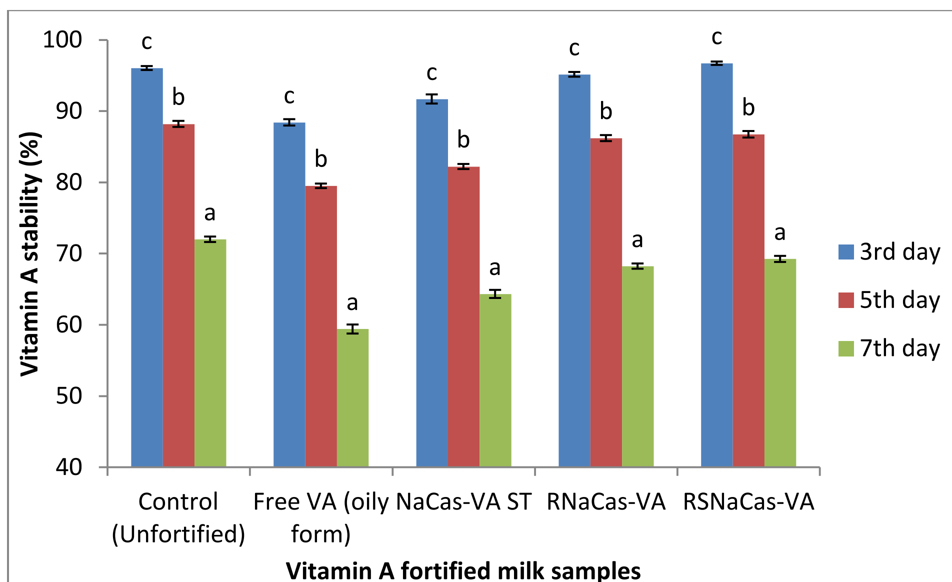
Table 4.67, figure 4.66 and 4.67 represent the vitamin A stability of milk stored in glass bottles at 4-7ºC. Control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) milk samples showed significant decrease ($p<0.05$) in vitamin A stability of milk stored in glass bottles on 3$^{rd}$, 5$^{th}$ and 7$^{th}$ day of storage (Fig 4.66). On 3$^{rd}$ day of storage, free vitamin A (oily form) fortified milk showed lowest vitamin A stability followed by NaCas-VA ST, RNaCas-VA, control (unfortified) and RSNaCas-VA. Control (unfortified) and RNaCas-VA complex fortified milk showed non significant difference ($p>0.05$) in vitamin A stability. However, RSNaCas-VA complex fortified milk showed slight but significant difference ($p<0.05$) in vitamin A stability as compared to control (unfortified) and RNaCas-VA complex fortified milk. On 5$^{th}$ day

of storage, free vitamin A (oily form) fortified milk showed lowest vitamin A stability followed by NaCas-VA ST, RNaCas-VA, RSNaCas-VA and control (unfortified) milk. Control (unfortified) milk showed slight but significant difference ($p<0.05$) in vitamin A stability as compared to RNaCas-VA and RSNaCas-VA complexes fortified milk. On 7th day of storage, free vitamin A (oily form) fortified milk showed lowest vitamin A stability followed by NaCas-VA ST, RNaCas-VA, RSNaCas-VA and control (unfortified) milk. RNaCas-VA and RSNaCas-VA complex fortified milk showed slight but significant difference ($p<0.05$) in vitamin A stability (Fig 4.67).

**Table 4.67: Effect of storage (4-7ºC) in glass bottles on vitamin A stability of milk**

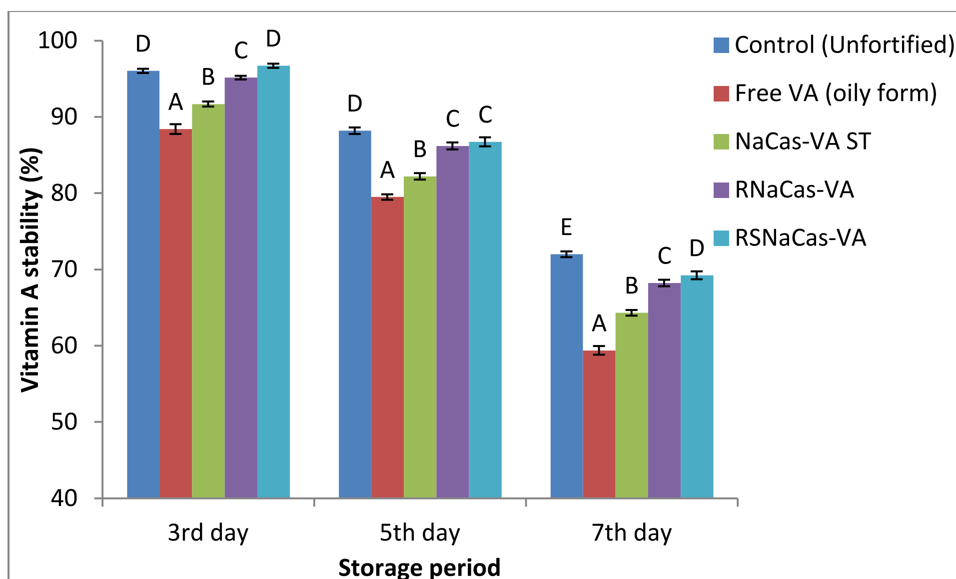
| Storage period (day) | Vitamin A stability (%) | | | | |
|---|---|---|---|---|---|
| | Control (unfortified) | Vitamin A fortified milk | | | |
| | | Free vitamin A (oily form) | NaCas-VA ST | RNaCas-VA | RSNaCas-VA |
| **3** | $96.03\pm0.28^{cD}$ | $88.40\pm0.64^{cA}$ | $91.69\pm0.33^{cB}$ | $95.14\pm0.24^{cC}$ | $96.70\pm0.27^{cD}$ |
| **5** | $88.19\pm0.43^{bD}$ | $79.49\pm0.36^{bA}$ | $82.21\pm0.42^{bB}$ | $86.19\pm0.46^{bC}$ | $86.73\pm0.59^{bC}$ |
| **7** | $71.99\pm0.38^{aE}$ | $59.39\pm0.57^{aA}$ | $64.31\pm0.37^{aB}$ | $68.23\pm0.43^{aC}$ | $69.23\pm0.52^{aD}$ |

Data are presented as means±SEM (n=3)
$^{a-b}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.
$^{A-B}$Means within rows with different superscript are significantly different ($p<0.05$) from each other.




$^{a-b}$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.

**Fig 4.66: Effect of storage (4-7°C) in glass bottles on vitamin A stability of milk**




[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.

**Fig 4.67: Comparison of vitamin A stability in control and vitamin A fortified milk samples as affected by storage (4-7$^oC$) in glass bottles**

Binding of free vitamin A (oily form) to milk protein improved the stability of vitamin A as compared to free vitamin A (oily form) (section 4.9.2). Control (unfortified) milk showed higher stability of vitamin A as compared to vitamin A fortified milk (containing NaCas-VA ST, RNaCas- VA, RSNaCas-VA and free vitamin A (oily form)). Our results are in accordance with Thompson and Erdody (1974), Bartholomew and Ogden (1990) who reported that indigenous vitamin A was more stable as compared to fortified vitamin A when exposed to light. Higher stability of vitamin A was observed in glass bottles as compared to LDPE pouches. Losses in LDPE pouches were partly due to the sorption of vitamin A by polyethylene pouches. Our results are in accordance with Kaushik *et al.* (2014b) who reported that vitamin $D_2$ fortified milk showed higher losses of vitamin $D_2$ in polyethylene pouches (8.66% loss on 7$^{th}$ day of storage) as compared to glass bottles (0.84% loss on 7$^{th}$ day of storage). Bhawana (2012) also reported that vitamin A fortified milk showed higher losses of vitamin A in polyethylene pouches (18.99% loss on 7$^{th}$ day of storage) as compared to glass bottles (11.51% loss on 7$^{th}$ day of storage). Paredes (1996) also indicated that the maximum sorption of vitamin A was 63% by low density

polyethylene (LDPE) plastic bottle and up to 55% by high density polyethylene (HDPE) plastic after one hour of contact with fortified skim milk.

Schroder *et al.* (1985) reported that oxidative reactions were controlled in glass container (if free oxygen was depleted), but they continued in cartons and high density polyethylene containers, which were more permeable to oxygen than glass.

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.

Bector and Rani (1998) 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. 3.5% loss 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.

#### 4.11.4 Evaluation of the effect of storage on vitamin A stability in sterilized milk stored in transparent glass bottles

Control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) sterilized milk samples showed significant decrease ($p<0.05$) in vitamin A content during storage in glass bottles at room temperature (37°C) for a storage period of 90 days (Table 4.68).

Table 4.69, figure 4.68 and 4.69 depict the stability of vitamin A in sterilized milk stored in glass bottles at 37°C. Control (unfortified), free vitamin A (oily form), NaCas-VA ST, RNaCas-VA and RSNaCas-VA complex fortified milk showed 75.52, 61.39, 66.79, 71.52 and 72.25% vitamin A stability after 15 day of storage and 12.60, 0.23, 2.76, 6.47 and 7.87% vitamin A stability after 90 day of storage. Vitamin

**Table 4.68: Effect of storage (37°C) on vitamin A content in sterilized milk stored in glass bottles**

| Storage period (day) | Vitamin A content (IU/L) | | | | |
|---|---|---|---|---|---|
| | Control (unfortified) | Vitamin A fortified milk | | | |
| | | Free vitamin A (oily form) | NaCas-VA ST | RNaCas-VA | RSNaCas-VA |
| **0** | $377.55\pm16.65^{f}$ | $1747.13\pm14.73^{e}$ | $2192.49\pm9.21^{g}$ | $2150.89\pm16.55^{g}$ | $2268.46\pm13.89^{g}$ |
| **15** | $284.98\pm11.12^{e}$ | $1073.93\pm48.17^{d}$ | $1464.34\pm13.31^{f}$ | $1538.48\pm27.98^{f}$ | $1639.09\pm17.42^{f}$ |
| **30** | $223.94\pm8.27^{d}$ | $567.80\pm43.34^{c}$ | $848.32\pm9.83^{e}$ | $1028.66\pm7.38^{e}$ | $1112.94\pm12.54^{e}$ |
| **45** | $145.64\pm6.05^{c}$ | $261.46\pm22.47^{b}$ | $470.89\pm5.28^{d}$ | $632.09\pm15.05^{d}$ | $708.20\pm6.36^{d}$ |
| **60** | $102.93\pm3.29^{b}$ | $147.66\pm14.61^{ab}$ | $299.34\pm9.38^{c}$ | $451.39\pm13.35^{c}$ | $505.19\pm5.93^{c}$ |
| **75** | $67.75\pm1.66^{a}$ | $59.40\pm2.82^{a}$ | $158.72\pm10.28^{b}$ | $275.58\pm10.74^{b}$ | $312.69\pm9.91^{b}$ |
| **90** | $47.44\pm0.56^{a}$ | $3.84\pm0.87^{a}$ | $60.62\pm4.76^{a}$ | $139.33\pm7.43^{a}$ | $178.45\pm7.90^{a}$ |

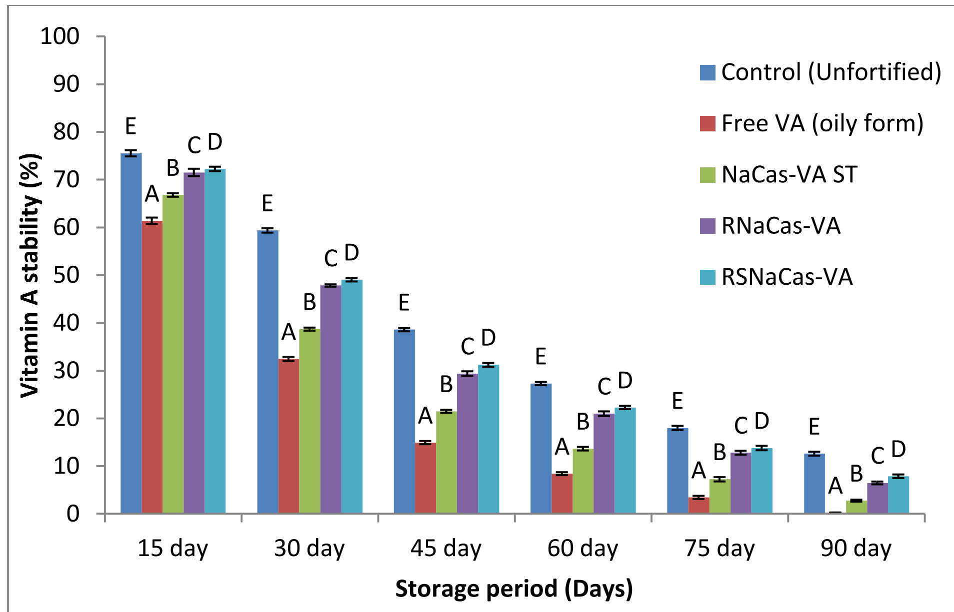
Data are presented as means±SEM (n=3)
$^{a\text{-}b}$Means within rows with different superscript are significantly different ($p<0.05$) from each other.

A stability in control (unfortified) and vitamin A fortified (containing NaCas-VA ST, RNaCas-VA, RSNaCas-VA and free vitamin A (oily form)) milk was significantly different ($p<0.05$) from each other during the storage period of 90 day. Free vitamin A (oily form) fortified milk showed lowest vitamin A stability followed by NaCas-VA ST, RNaCas-VA, RSNaCas-VA and control (unfortified) milk. RSNaCas-VA and RNaCas-VA complex fortified milk showed higher vitamin A stability as compared to NaCas-VA ST (Fig 4.68). Binding of free vitamin A (oily form) to milk protein improved the vitamin A stability. Control (unfortified) and vitamin A fortified milk samples showed significant decrease ($p<0.05$) in vitamin A stability during the storage period of 90 days (Fig 4.69).

**Table 4.69: Effect of storage (37°C) on vitamin A stability in sterilized milk stored in glass bottles**

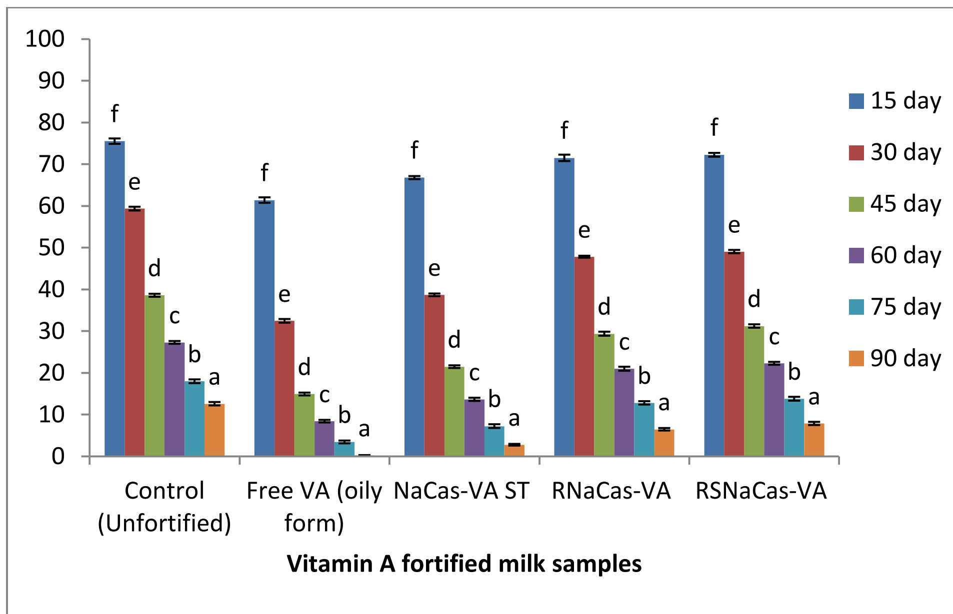
| Storage period (day) | Vitamin A content (IU/L) | | | | |
|---|---|---|---|---|---|
| | Control (unfortified) | Vitamin A fortified milk | | | |
| | | Free vitamin A (oily form) | NaCas-VA ST | RNaCas-VA | RSNaCas-VA |
| **15** | $75.52\pm0.65^{fE}$ | $61.39\pm0.66^{fA}$ | $66.79\pm0.36^{fB}$ | $71.52\pm0.77^{fC}$ | $72.25\pm0.46^{fD}$ |
| **30** | $59.35\pm0.45^{eE}$ | $32.45\pm0.44^{eA}$ | $38.69\pm0.32^{eB}$ | $47.83\pm0.24^{eC}$ | $49.06\pm0.39^{eD}$ |
| **45** | $38.59\pm0.35^{dE}$ | $14.92\pm0.34^{dA}$ | $21.48\pm0.32^{dB}$ | $29.38\pm0.48^{dC}$ | $31.22\pm0.40^{dD}$ |
| **60** | $27.29\pm0.33^{cE}$ | $8.41\pm0.31^{cA}$ | $13.65\pm0.37^{cB}$ | $20.98\pm0.48^{cC}$ | $22.27\pm0.35^{cD}$ |
| **75** | $17.98\pm0.45^{bE}$ | $3.44\pm0.33^{bA}$ | $7.24\pm0.45^{bB}$ | $12.81\pm0.41^{bC}$ | $13.79\pm0.47^{bD}$ |
| **90** | $12.60\pm0.41^{aE}$ | $0.23\pm0.06^{aA}$ | $2.76\pm0.21^{aB}$ | $6.47\pm0.30^{aC}$ | $7.87\pm0.38^{aD}$ |

Data are presented as means±SEM (n=3)
$^{a\text{-}b}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.
$^{A\text{-}B}$Means within rows with different superscript are significantly different ($p<0.05$) from each other.




[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.

**Fig 4.68: Comparison of vitamin A stability in control and vitamin A fortified sterilized milk samples as affected by storage (37ºC) in glass bottles**




[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.

**Fig 4.69: Vitamin A stability (%) in sterilized milk as affected by storage in glass bottles at 37°C**

Our results are in accordance with McCarthy *et al.* (1986) who reported that vitamin A fortified ultra high temperature (UHT) processed milk showed significant losses of retinyl palmitate during storage at 23±2°C for 15 weeks. Lau *et al.* (1986) reported the vitamin A content in UHT processed milk decreases rapidly during first 2 week of storage (26°C) and then stabilized. Maguer and Jackson (1983) reported that storage of UHT processed milk at 35°C adversely affected its vitamin stability, notably after 12 week of storage.

### 4.12 *In-vitro* bioavailability of vitamin A from milk protein-Vit A complexes and free vitamin A (oily form) fortified milk

*In-vitro* bioavailability of vitamin A was lowest for free vitamin A (oily form) fortified milk. Milk fortified with various milk protein-Vit A complexes (NaCas-VA ST, RNaCas-VA and RSNaCas-VA) showed significantly higher ($p<0.05$) *in-vitro* bioavailability of vitamin A as compared to free vitamin A (oily form) fortified milk. Milk protein-Vit A complexes (NaCas-VA ST, RNaCas-VA and RSNaCas-VA) fortified and control (unfortified) milk showed non significant difference ($p>0.05$) in bioavailability of vitamin A (Table 4.70). However, control (unfortified) milk contain lower concentration of vitamin A (400 IU/L) as compared to milk protein-Vit A complex fortified milk (2400 IU/L), hence, vitamin A was more bioavailable through milk protein-Vit A complex fortified milk.

**Table 4.70: *In-vitro* bioavailability of vitamin A from milk protein-Vit A complexes and free vitamin A (oily form)**

| Milk samples | | *In-vitro* bioavailability (%) |
|---|---|---|
| Control (unfortified) | | $80.21±0.32^{b}$ |
| Vitamin A fortified milk | Free vitamin A (oily form) | $63.92±0.18^{a}$ |
| | NaCas-VA ST | $80.14±0.21^{b}$ |
| | RNaCas-VA | $80.79±0.31^{b}$ |
| | RSNaCas-VA | $80.84±0.24^{b}$ |

Data are presented as means±SEM (n=3)
$^{a-b}$Means within columns with different superscript are significantly different ($p<0.05$) from each other.

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.

# CHAPTER –5

# Summary and Conclusion

# SUMMARY AND CONCLUSION

The nutritional status of the population is one of the most important factor in determining the quality and productivity of a population, which in turn will affect national productivity. In the long run, a good nutritional status contributes to the social and economic development of a nation. A significant proportion of the World's population suffers from, or is at a risk from deficiencies of vitamins and minerals, referred as micronutrients. Adequate intake and availability of these dietary essential vitamins and minerals are closely related to the survival, physical and mental development, general health and overall well-being of individual and population. Among micronutrients deficiencies, iron, vitamin A, iodine and vitamin D deficiencies are of greatest public health significance. VAD affecting more than 100 million children in 75 countries; can lead to partial or total blindness.

Now days, due to the increasing interest in leading a healthy life, the use of fortified food is presented as an alternative source to overcome nutritional deficiencies. Recently, FSSAI approved the fortification of toned/double toned/skimmed milk with vitamin A and vitamin D. However, incorporation of fat soluble vitamins into low fat foods is still a challenging task as these are highly sensitive to oxidation, hence require stabilization. Among the various technologies explored, binding of vitamin to milk protein may improve the solubility and stability of fat soluble vitamins. Different milk protein (reassembled casein micelles, native (unmodified) milk protein and modified (succinylated) milk protein)-Vit A complexes were prepared and complexes for further studies were selected on the basis of vitamin A binding ability of milk proteins and solubility of milk protein-Vit A complexes. Physicochemical characterization, stability and *in-vitro* bioavailability of milk protein-Vit A complexes were reported. Finally, milk fortified with milk protein-Vit A complexes was evaluated for sensory, physicochemical properties. I*n-vitro* analysis of fortified milk was also carried out to determine the bioavailability of vitamin A. The main findings of the present investigation have been listed as follows:

1. Native milk protein-Vit A complexes were prepared using NaCas, WPC, MPC and modified milk protein-Vit A complexes were prepared using

SNaCas and SMPC. Reassembled milk protein-Vit A complexes were prepared using NaCas and SNaCas.

2. ß-CN was prepared using calcium caseinate and skim milk. Yield of ß-CN prepared by different methods was very less, hence was not considered for the further evaluation.
3. Method for the preparation of succinylated milk proteins has been standardized. The level of succinic anhydride required for the succinylation of NaCas and MPC was optimized on the basis of maximum degree of succinylation (optimized succinic anhydride level to achieve maximum degree of succinylation was 3.2 moles of succinic anhydride/mole of lysine content for both NaCas and MPC).
4. Native and modified milk protein-Vit A complexes were prepared using both stirring and sonication.
5. Method was optimized for the preparation of reassembled milk protein-Vit A complexes.
6. Analytical conditions were optimized for the extraction and estimation of total vitamin A on HPLC.
   - 6.1 Sample was treated with 2.5% $NH_3$ and 0.1 N HCl during saponification.
   - 6.2 Saponification was done at 70°C for 30 min.
   - 6.3 Chloroform and methanol was selected as solvents for extraction of vitamin A from saponified sample.
   - 6.4 Mobile phase consisted of methanol: acetonitrile: water (49.5: 49.5: 1) was used for vitamin A analysis at a flow rate of 1.0 ml/min. Column temperature was maintained at 40°C. The sample volume injected was 20 µl and a PDA detector at 325 nm was used to monitor the eluate.
7. Centrifugation, UF, isoelectric precipitation and ammonium sulphate precipitation method were tried for estimation of unbound vitamin A. Among all these methods, ammonium sulphate precipitation method gave optimum result.

8. Milk protein-Vit A complexes i.e. NaCas-VA ST, SNaCas-VA ST, RNaCas-VA and RSNaCas-VA were selected from both native and succinylated milk protein-Vit A complexes for further evaluation on the basis of vitamin A binding ability of milk proteins and solubility of milk protein-Vit A complexes.
9. 5 gm of milk protein was able to bind with 4.5 lakh IU of vitamin A in RNaCas-VA complexes. This optimized concentration of vitamin A was used for preparation of other selected milk protein-Vit A complexes.
10. Physicochemical characteristics such as particle size, zeta potential, turbidity analysis and tryptophan intensities confirmed structural modification of both native (NaCas-VA ST, RNaCas-VA) and succinylated (SNaCas-VA ST, RSNaCas- VA) proteins upon complex formation with vitamin.
11. Binding of vitamin A to milk protein reduced the turbidity caused by vitamin A, however, the particle size and zeta potential of milk protein increased.
12. Microstructure details of NaCas (spray dried) showed uniform spherical structure, however, other milk protein and milk protein-Vit A complexes (freeze dried) showed broken glass and flaky structure. Tiny particles were observed on the surface of reassembled protein and reassembled protein-Vit A complexes.
13. Binding of vitamin A to milk protein did not have an influence on the electrophoretic mobility and elution profile of RP-HPLC.
14. Milk protein-Vit A complexes and free vitamin A (oily form) showed lowest vitamin A stability at 37°C followed by 4°C and -20°C.
15. Milk protein-Vit A complexes and free vitamin A (oily form) showed lower vitamin A stability in microcentrifuge tubes (virgin polypropylene) as compared to aluminium laminate pouches (Polyethylene terephthalate or nylon/Aluminium foil/polypropylene, thickness 0.11 mm).
16. Stability of milk protein-Vit A complexes were ascertained at three different pH (3.0, 5.0 and 7.0).
17. All milk protein-Vit A complexes showed significantly higher ($p<0.05$) *in-vitro* bioavailability of vitamin A as compared to free vitamin A (oily form).

18. On the basis of storage and pH stability, NaCas-VA ST, RNaCas-VA and RSNaCas-VA complexes were selected for fortification of vitamin A in milk.
19. Fortification of milk with free vitamin A (oily form) and milk protein-Vit A complexes did not have a significant influence on sensory and physicochemical properties of fortified milk.
20. Vitamin A stability was lowest in sterilized milk followed by boiled and pasteurized milk. The stability of vitamin A was lowest for free vitamin A (oily form) fortified milk followed by NaCas-VA ST, RNaCas-VA, RSNaCas-VA complex fortified milk and control (unfortified) milk.
21. Pasteurized milk fortified with vitamin A showed higher stability in transparent glass bottles as compared to LDPE pouches during storage at 4-7°C for 7 days, however, higher stability of vitamin A was observed in LDPE pouches as compared to transparent glass bottles during exposure to different light intensities i.e. 1485, 2970 and 4455 lux.
22. Milk fortified with various milk protein-Vit A complex showed higher *in-vitro* bioavailability of vitamin A as compared to free vitamin A (oily form) fortified milk.
23. Among the various milk protein-Vit A complexes, RSNaCas-VA complex showed highest stability and *in-vitro* bioavailability of vitamin A ($p<0.05$).

In conclusion, the present work led to the development of method for preparation of different milk protein (both native and succinylated)-Vit A complexes. Analytical conditions were also standardized for extraction and estimation of total and unbound vitamin A using HPLC. The obtained milk protein-Vit A complexes were rich in protein and vitamin A and hence could be used as a vitamin A fortificant with better solubility, stability and bioavailability than free vitamin A (oily form). Evaluation of milk fortified with these milk protein-Vit A complexes suggested that these complexes could be added to food products with minimal effect on sensory acceptability, physicochemical characteristics and product shelf life. These complexes can also be dry blended into whole milk or skim milk powder. Further, the powdered milk protein-Vit A complexes can also be sold as ingredients for incorporation into other food products e.g. infant formula, cheese, yoghurt, ice cream

etc. The present work involved the preparation of various milk protein-Vit A complexes using freeze dryer, which consumed lot of time to obtain the final product. Therefore, to overcome this limitation attempts should be made to manufacture milk protein (both native and succinylated protein)-Vit A complexes using spray dryer.

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# Annexures

# Annexure I

## SENSORY CARD FOR MILK

**Date:** **Storage time:**

**Name of panelist :**

**Objective : To evaluate vitamin A fortified milk for its sensory attributes**

**A) Assign score for each sample for different characteristics**

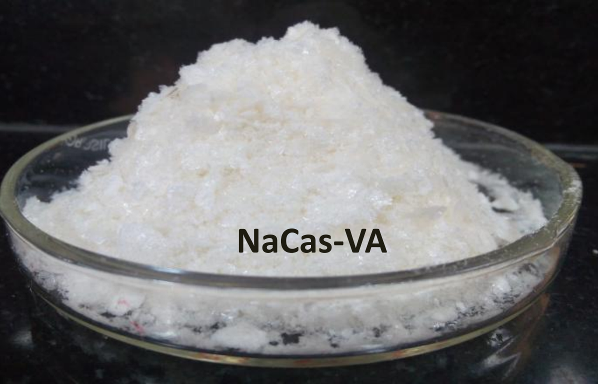
| Characteristics | Maximum score | Sample score 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|
| 1. Colour and appearance | 10 | | | | | |
| 2. Odour | 20 | | | | | |
| 3. Taste | 40 | | | | | |
| 4. Mouthfeel | 30 | | | | | |

**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, Off flavour | 5 | 10 | 20 |
| 4. Mouthfeel | Watery, heavy, curdy, chalky | 5 | 10 | 15 |

Comments:

Signature



Milk protein–Vit A complexes (both native and succinylated ) could be used as an organic fortificant with better solubility, stability and bioavailability

# OUT COME OF THE RESEARCH

Milk protein-Vit A complex development allows addition of vitamin A to most food products with minimal effect on sensory, physicochemical characteristics and product shelf life

# Chitra Gupta

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## Educational credentials

| Degree | Spécialisation | Institution / Location | OGPA/Percent | Year |
|---|---|---|---|---|
| Ph.D. | Dairy Chemistry | National Dairy Research Institute, Karnal | 8.6/10 | 2013-2017 |
| M.Tech. | Dairy Chemistry | National Dairy Research Institute, Karnal | 8.0/10 | 2011-2013 |
| B.Tech. | Dairy Technology | National Dairy Research Institute, Karnal | 8.1/10 (**Gold Medalist**) | 2007-2011 |
| 10+2$^{th}$ | PCM | Gopal Academy Senior Sec. School, Alwar (Raj.) | 79.23% | 2005-2006 |
| 10$^{th}$ | General | Shri Oswal Jain Sec. School, Alwar (Raj.) | 81.17% | 2003-2004 |

## Research Work

- **M.Tech.** research work on **"Development and evaluation of microencapsulated iron fortified yoghurt".** Work was planned to develop iron microcapsules and its fortification in plain set yoghurt. Iron fortified yoghurt was also evaluated for it physicochemical, rheological, sensorial and microbiological parameters.

## Academic Achievements

**Awards**

- Recipient of **Best Poster Award** in National Conference on "Food Processing and Technology: Current Status and Future Prospects (NCFPT-2016)" organized by Shoolini University, Solan (HP) 25-26$^{th}$ February, 2016.
- Recipient of **II$^{nd}$ Best Poster Award** in 44$^{th}$ Dairy Industry Conference on "Make in India: Dairying 2030" organised by ICAR- National Dairy Research Institute, 18-20$^{th}$ February, 2016.
- Recipient of **Appreciation certificate for outstanding M.Tech research work** on "Development and evaluation of microencapsulated iron fortified yoghurt" in the field of **Dairy Processing** during 12$^{th}$ convocation of NDRI, 14$^{th}$ Feb 2014.
- Recipient of **Gold Medal (2007-2011)** as University topper in B.Tech. (Dairy Technology) from Hon'ble M.S. Swaminathan at NDRI, Karnal
- Recipient of **Prof. M.R. Srinivasan Memorial award** as university topper in B.Tech. (2011)

**Examination Qualified**

- Qualified **Senior Research Fellowship (PGs)-2014 with fellowship** in Dairy Chemistry.
- Qualified **ICAR-National Eligibility Test** examination-2013 **(NET- 2013)** conducted by Agricultural Scientists Recruitment Board, New Delhi with 69% marks.
- Qualified **GATE-2011** with all India rank 165.

## Professional synopsis

**Research papers entitled:**

1. Iron microencapsulation with blend of Gum arabic, Maltodextrin and Modified starch using modified solvent evaporation method – Milk Fortification. ***Food Hydrocolloids***, 2015; 43, 622-628. (Impact factor **4.280,** NAAS rating **10.28**)
2. Development and evaluation of iron microencapsules for milk fortification. ***CyTA-Journal of Food***, 2015; 13(1), 116-123. (Impact factor **0.495,** NAAS rating **6.5**)
3. Effect of fat content on sensory and physico-chemical properties of laboratory-pasteurised calcium and vitamin D fortified mixture of cow and buffalo milk. ***International Journal of Dairy Technology***, 2015; 68(1), 135-243. (Impact factor **2.297,** NAAS rating **7.1***)*

**Book chapter entitled:**

1. **Fortification of Milk and Milk products for Value Addition.** All India Dairy Business Directory (Dairy Year Book), A mini encyclopedia, 2014; Vol 1 (pp 105-109).

**Oral presentation on:**

1. "Sensory and physicochemical properties of yoghurt as affected by iron microencapsules fortification" in the $6^{th}$ International conference on "Fermented foods health status and social well being" held at AAU, Anand, 6-7 December, 2013.

**Abstract and posters published in abstract book of various conferences:**

1. Comparison of different method for iron microencapsulation
2. Iron microencapsulation with blend of gum arabic, maltodextrin and modified starch (HiCap 100) using modified solvent evaporation method
3. Preparation of Iron Microcapsules by modified Solvent Evaporation Method
4. Microencapsulation of iron with blend of gum arabic, maltodextrin and modified starch
5. Comparison of different hydrating media on encapsulation efficiency of iron \microencapsules
6. Sensory and physicochemical properties of yoghurt as affected by iron microencapsules fortification
7. Polyglycerol monostearate and lecithin as coating material for microencapsulation of iron
8. Evaluation and acceptability of lactose-iron complex fortified milk
9. Functional property , antioxidant activity, mineral analysis and *in vitro* bioavailability of *Cucumis melo* and *Citrullus vulgaris* seed flour
10. Antioxidant activity and mineral content of Pistacia vera and Prunus dulcis
11. Effect of developed acidity and neutarlization on protein oxidation in milk and khoa prepared from cow and buffalo milk
12. Antioxidant activity and mineral content of seed extracts of Cucumis melo and Citrullus vulgaris
13. Determination of vitamin $D_2$ and calcium bioavailability from fortified milk