Abstract

Liu, Ying. β-lactoglobulin complexed vitamins A and D in skim milk: shelf life and bioavailability. (Chair: Jonathan C. Allen)

As reduced fat dairy products became popular in the U.S., the reduction of vitamins A and D in these products rendered a nutritional concern. β-lactoglobulin has been reported capable of binding fat-soluble vitamins. In order to justify the potential of β-lactoglobulin as a stable and protective carrier for the vitamins, stability and bioavailability of β-lactoglobulin complexed vitamins A and D in skim milk were investigated in this study.

The first objective of this research was to observe the stability of vitamin A palmitate and vitamin D_3 in fortified skim milk during storage. The fortifiers included regular oil-based vitamins, Roche dry vitamins, and spray-dried and freeze-dried protein-based vitamins. Milk was stored in different packaging materials including glass test tubes, plastic bottles, and paperboard cartons. The effect of High Temperature Short Time (HTST) and Ultra High Temperature (UHT) pasteurizations on shelf life was also studied. Vitamin A palmitate and vitamin D_3 were extracted by organic solvents and assayed by High Performance Liquid Chromatography (HPLC). Results showed that vitamin D_3 was relatively stable in all milks during the 4 weeks of shelf life. Vitamin A concentrations remained stable in most HTST milk except for those fortified with Roche dry vitamin A. A significant decrease in retinyl palmitate was detected in UHT protein-based vitamin-fortified skim milk. The second objective was to test the ability of different forms of fortified skim milk to boost vitamin levels in vitamin-depleted rats. Serum, liver and bone tissues were assayed for both vitamins and calcium. Data indicated that oil-based vitamin A-fortified skim milk was as efficient as positive AIN-93G pelleted rodent diet in raising the vitamin A levels in the tissues and protein-based vitamin D_3 fortified skim milk most effectively boosted the serum 25-OH D level.

In conclusion, β-lactoglobulin complexed vitamin A palmitate is not an appropriate fortifier for UHT skim milk while β-lactoglobulin complexed vitamin D_3 is a more stable and effective fortifier than the regular oil-based fortifier.

Beta-Lactoglobulin Complexed Vitamins A and D in Skim Milk: Shelf Life and Bioavailability

By

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Biography

Ying Liu was born on Nov. 29th, 1971 in P.R.China. She is the oldest daughter of Mrs. Xinxin He and Mr. Rongzong Liu. Ying enjoyed her childhood with two lovely younger sisters, Song and Rachel. In 1989, she went to Jiangxi Medical College for her undergraduate study majoring in Public Health, where she won the First Scholarship for five years. Then she decided to have some exploration in the field of molecular biology. In 1994, she was accepted by Medical School of Shantou University and started her master program in Biochemistry. Ying was awarded with the Lee Ka Shing Scholarship during the three-year study. After graduation, she taught for two years in the same institute. At that time she met her husband, Dr. Bin Yu and they got married in May 1998. Their daughter was born in April 1999.

A year after she came to the U.S. with her husband, Ying attended the Nutrition Program at NCSU and worked with Dr. Jonathan C. Allen towards her Ph.D. degree for the next four years. Ying received NC Institute of Nutrition Fellowship twice from NCSU. And she is a student member of the honor society of agriculture, *Gamma Sigma Delta* since April 2002.

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Chapter 1 Introduction

Vitamin A is an essential micronutrient in the diet for vision, growth, tissue, reproduction, maintenance of immune system, and other functions. Although Americans are at little risk of developing a deficiency, vitamin A deficiency is one of the major public health problems in developing countries. Recent studies demonstrated higher prevalence of sub-clinical vitamin A deficiency in preschool children who do not eat enough vegetables and patients with chronic gastrointestinal diseases (Russell, 2000). It is well established that vitamin D plays important roles in maintaining calcium homeostasis, bone growth and health. Other potential functions of vitamin D include development, growth, and prevention of cancer and other chronic diseases such as diabetes (Holick, 2003). Though called the "sunshine" vitamin, dietary sources of vitamin D may be necessary because of insufficient photosynthesis of vitamin D due to inadequate skin exposure to sunlight. Vitamin D deficiency (rickets in children and osteomalacia in adults) is making resurgence in breast-fed African American children (Kreitter, 2000) and even in active, young adults (Outila, 2001).

In the U.S., one of the major sources of dietary vitamins A and D is dairy products fortified with retinyl palmitate and vitamin D_3 (cholecalciferol). Currently the acceptable range for vitamin D is 400-600 IU/qt, and for vitamin A it is 2000-3000 IU/qt in fortified milk (PHS/FDA, 1999). However, multiple studies found that a high percentage of fortified milk products failed to meet label claims: most of the milk out of compliance was under-fortified (Holick, 1992; Nichols, 1992). Loss of vitamin often occurred during processing and storage (Tanner, 1988). Vitamin D_3 is relatively stable, while the degradation of vitamin A

occurs in the presence of light, oxygen, and free radicals mainly through reactions involving the unsaturated isoprenoid side chain by either autoxidation and/or geometric isomerization. Another cause of loss in skim milk packaged in polyethylene containers was reported to be due to sorption of the vitamin to the polyethylene material in the absence of lipids (Paredes, 1996). Under-fortification of the vitamins in skim milk and other dairy products has rendered potential fat-soluble vitamin deficiencies a public health concern.

Beta-lactoglobulin (BLG) has been reported capable of binding fat-soluble compounds, including vitamins A and D_3. Therefore it is a potential carrier to protect vitamins from oxidation, especially in skim and low-fat dairy products. Data from fluorescence spectroscopy suggest that BLG is capable of binding at least one mole of retinyl palmitate and vitamin D_3, per mole (Wang, 1997). Furthermore, BLG may also facilitate absorption of retinol in the intestine (Said, 1989). Whey protein isolate (WPI, mainly BLG) has also been used as an efficient encapsulation agent by spray drying. Oxidation of anhydrous milk fat was significantly limited by micro-encapsulation in WPI (Moreau, 1996).

In this study, stability and bioavailability of β-lactoglobulin complexed vitamins A and D in skim milk were investigated to justify the potential of β-lactoglobulin as a stable and protective carrier for the vitamins. The first purpose was to prepare spray-dried and freeze-dried protein-vitamin complexes, use them to fortify skim milk, test the stability of vitamins in skim milk during the shelf life, and compare that with other fortifiers after HTST or UHT pasteurization procedures in plastic or glass containers. The influence of pasteurizations, containers, and carriers on shelf-life stability of vitamins was studied. The second purpose was to study the capacity of protein-based and oil-based vitamin- fortified UHT processed skim milk to boost vitamin levels in depleted rats. AIN-93G pelleted rodent diet and non-

fortified skim milk with a vitamin-A and D-deficient diet served as positive and negative controls, respectively. Liver and serum vitamin levels were measured to indicate vitamin A status and serum 25-OH D was used as the indicator of vitamin D status. Serum and bone calcium, bone ash percentage and a three-point bending test were also performed.

This dissertation first reviews metabolism and biological functions of retinyl palmitate and vitamin D_3, molecular properties of BLG and its binding characteristics with vitamins A and D, milk fortification and compliance, and HPLC methodology for vitamin assays. The main body of this dissertation describes preparation of protein-vitamin complexes, shelf life studies, and the bioavailability study. Methods, results, discussion and conclusions were separately included for each experiment.

Chapter 2 Literature Review

2.1 Vitamin A

2.1.1 Source, absorption, transport, storage and excretion of vitamin A

Vitamin A refers to pro-vitamin A, certain carotenoids and preformed retinoids, plus their metabolites (Fig. 2.1). Retinoids are the biologically active forms of vitamin A with four isoprenoid units, including retinol, retinal, and retinoic acid (Olson, 1994b). In animal foods such as egg yolk, butter, dairy products and liver, preformed vitamin A is present as retinol and retinyl esters (such as retinyl palmitate) that have a fatty acid attached to retinol (Fennema, 1996). Carotenoids are dietary precursors of retinol in fruits and vegetables that range in color from yellow to orange to red such as squash, carrots, tomatoes, etc. All green vegetables contain some beta-carotene equivalents, though the pigment is masked by chlorophyll (Groff, 1995). In the typical North American diet, milk and dairy products are major sources of vitamin A, whereas pro-vitamin A predominates in the diet of developing countries.

The release of vitamin A from food requires bile, digestive enzymes from the pancreas and the intestinal tract, and integration into micelles. Patients with chronic fat-malabsorption syndromes have trouble absorbing vitamin A and may develop a deficiency. Most of the preformed vitamin A in the diet is in the form of retinyl esters (RE); retinyl palmitate is the most common ester in food. Pancreatic nonspecific lipase, cholesterol ester hydrolase, and esterases from the intestinal brush border hydrolyze retinyl and carotenoid esters (Ong, 1993; Groff, 1995). The released β-carotene and retinol then form a micellar solution along with other fat-soluble food components, and diffuse through the glycoprotein layer into the enterocyte (Blomhoff, 1994). β-Carotene is transformed into retinal, and reduced to retinol

within the intestinal cell. Then retinol is re-esterified in the intestinal cell mainly through the pathway involving cellular retinol-binding protein (CRBP-II). Lecithin retinol acyl transferase (LRAT) recognizes the complex retinol-CRBPII and synthesizes retinyl esters (mainly retinyl palmitate). The minor pathway involves binding of retinol to a non-specific cellular protein, with subsequent re-esterification by acyl CoA retinol acyl transferase (ARAT) (Groff, 1995). Chylomicrons incorporate and deliver retinyl esters to the liver as chylomicron remnants formed after hydrolysis of triacylglycerol.

In liver parenchymal cells, lysosomes engulf the chylomicron remnants and an acidic hydrolase releases retinol to bind with cellular retinol-binding protein (CRBP). The holo-CRBP serves as a substrate for retinol esterification catalyzed by LRAT or ARAT (Ross, 1993). A neutral bile salt-independent RE hydrolase (bsiREH) catalyzes RE mobilization. Apo-CRBP controls retinol metabolism by inhibiting LRAT and increasing the rate of hydrolysis via the bsiREH. Holo-CRBP also serves as substrate for retinal formation, catalyzed by retinol dehydrogenase, which catalyzes the irreversible and possible rate-determining conversion of retinal to retinoic acid (RA). The presence of CRBP potentially prevents enhanced enzymes access to retinol. About 90% of the vitamin A is stored with other lipids in liver stellate cells as retinyl esters (primarily retinyl palmitate, but also as retinyl stearate, oleate and linoleate) (Groff, 1995).

When needed, retinol is mobilized from the liver by a hydrolase, transported to endoplasmic reticulum, bound to retinol-binding protein (RBP) and secreted from the liver to the plasma. In the plasma, the complex retinol-RBP is bound to transthyretin (TTR). TTR is also called prealbumin and binds one thyroxine (T_4) per tetramer. RBP and TTR serve to make retinol more soluble in plasma and protect it from being filtered from blood by the

kidneys. Uptakes of retinol by target cells are mediated by specific cell surface receptors that recognize RBP and internalized retinol, not RBP (Creek, 1993 and Sundaram, 1998). Once in the cell, retinol is bound with various proteins such as CRBP (Ong, 1994). Organs that take up retinol include adipose, skeletal muscle, kidney, eyes, etc. Retinol conversion to retinal is reversible, while the oxidation of retinal to retinoic acid is irreversible. The major catabolic pathway of retinoic acid is oxidation to 4-hydroxyl retinoic acid and other metabolites in a NADPH-dependent reaction. About 70% of vitamin A metabolites are conjugated to glucuronide and excreted via the bile into feces. Some is lost in the urine (Groff, 1995).

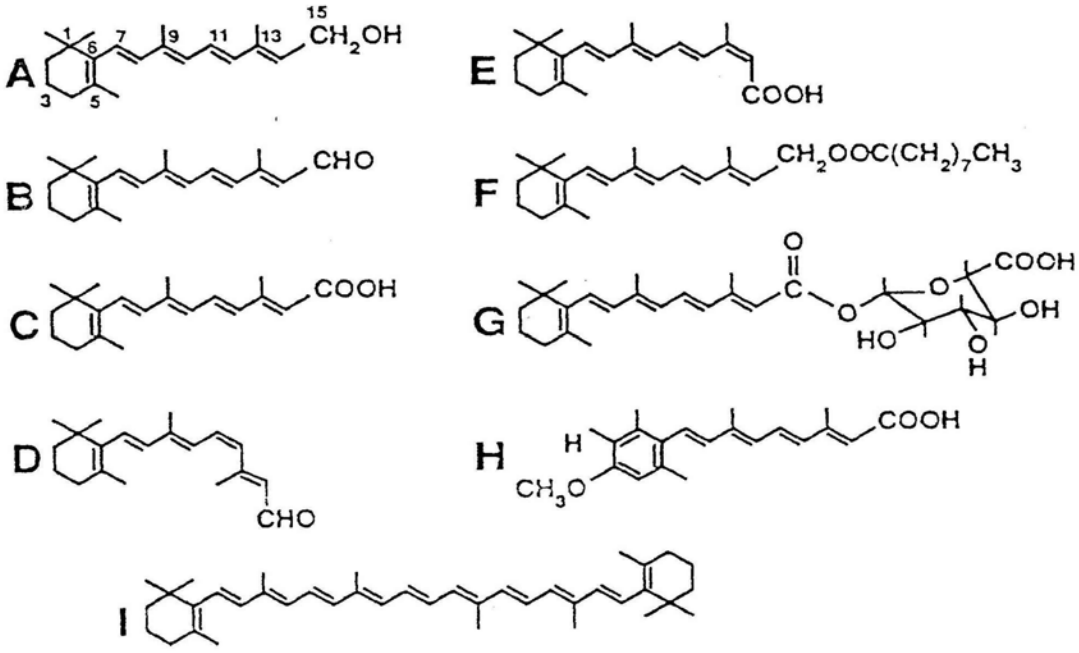

Figure 2.1 Chemical formulas of major retinoids and β-carotene

A, all-trans retinol; B, all-trans retinal; C, all-trans retinoic acid; D, 11-cis retinal; E, 13-cis retinoic acid; F, all-trans retinyl palmitate; G, all-trans retinoyl β-glucuronide; H, the trimethyl methoxyphenol analog of all-trans retinoic acid (etretin, acitretin); I, all-trans β-carotene.

2.1.2 Functions of vitamin A

Vision

Vitamin A is required for normal vision both as retinal in the retina of the eye, to turn visual light into nerve signals to the brain, and retinoic acid to maintain normal differentiation of the cells that make up the various structures of the eye, such as the cornea and rod cells. In the rods, 11-cis-retinal binds to opsin to form the visual pigment called rhodopsin. The absorption of a photon of light catalyzes the photo-isomerization of 11-cis-retinal to all-trans retinal, causing the release of 11-cis-retinal from opsin (bleaching process). The isomerization event leads to a cascade of biochemical events, which trigger a change in ion permeability of the photoreceptor cells, initiating a signal to neuronal cells that communicate with the brain's visual cortex. During exposure to bright light, rhodopsin is completely activated and cannot respond to more light until it returns to its resting state by enzymatic regeneration. If there is a limited amount of rhodopsin, it is difficult to adapt to seeing in dim light, which is known as night blindness. In order to keep the visual process functioning, the 11-cis-retinal must be regenerated by converting all-trans retinal to retinol, then to 11-cis-retinol, and finally back to 11-cis-retinal. This process takes several minutes. Some retinal is lost from the cycle, so there is a pool of retinyl esters in the eye to keep a supply of vitamin A. Should the pool of vitamin A be low, the process of dark adaptation is slowed down (Ross, 1993).

Cellular growth and differentiation

Since the late 1980s, there has been an explosion of information on the molecular mechanisms and functions of vitamin A (Clagett-Dame, 2002). In general, retinoic acid plays important roles in gene expression, cell differentiation and proliferation through reacting with the retinoic acid receptors, Retinoic Acid Receptors (RAR; α, β, and γ) and Retinoids X Receptors (RXR; α, β, and γ), which are part of the family of steroid and thyroid hormone nuclear transcription factors (Chambon, 1996). All-trans retinoic acid and 9-cis-retinoic acid bind with RAR and RXR, which then bind to specific DNA response elements, resulting in activation of specific genes and transcription of mRNA (Lee, 1997). In contrast to the steroid hormone receptors that function predominantly as homodimers, RARs and thyroid hormone receptors require interaction with RXR for efficient DNA binding and transactivation (Zhang, 1993). RXR can also form homodimers that recognize a subset of retinoic acid responsive elements (RAREs) in the presence of their specific ligand such as 9-cis RA. RXR homodimers or heterodimers formed with Peroxisome Proliferator Activated Receptors (PPAR), Vitamin D Receptor (VDR), Thyroid Receptor (TR), or RAR positively regulate gene expression through Hormone Response Elements (HREs) composed of tandem repeat sequences spaced by 1, 2, 3, 4, or 5 nucleotides. In addition, RXR forms heterodimers with Chicken Ovalbumin Upstream Promoter Transcription Factor (COUP-TF) and RAR, which negatively regulates gene expression through HREs composed of tandem repeats spaced by a single nucleotide. In general, retinoic acid is necessary for reproduction, growth and development, and the structure and normal function of epithelial cells in many organs such as gastrointestinal tract and lungs (Hadi, 2000). Retinoic acid also controls the production of glycoproteins, which are important in cell interaction, recognition and aggregation.

Other functions

Vitamin A seems to influence immunity through cell-mediated and antibody-mediated responses, such as macrophage and natural killer cell activity, and growth and differentiation of B-lymphocytes. In addition, nonspecific immunity, the maintenance of epithelial tissues, and mucus production all act as powerful deterrents to invading pathogens (Ross, 1993). Although many animal studies using natural and synthetic retinoids have shown a reduction in various types of cancer, such effects with long-term use of retinoids in humans has not been demonstrated. Possible mechanisms that vitamin A could use to prevent cancers include the fundamental role of retinoic acid in cell differentiation, inhibition of proliferation, induction of programmed cell death, and the antioxidant capability of carotenoids such as lycopene. Carotenoids may also play a role in preventing cardiovascular diseases through the antioxidant function (Pryor, 2000).

2.1.3 Deficiency, toxicity, and RDA for Vitamin A

Night blindness is a common early symptom of vitamin A deficiency, when the retinol in blood plasma is insufficient to replace the retinal lost during the visual cycle. Xerophthalmia, marked by dryness of the cornea and eye membranes, results from lack of mucus production by the eye and vulnerability of the eye to surface dirt and bacterial infections as vitamin A deficiency worsens. Xerophthalmia causes irreversible blindness in millions of people worldwide. Vitamin A deficiency also causes follicular hyperkeratosis, in which keratinized cells replace the normal epithelial cells in the underlying skin layer. A decrease in appetite and poor growth are also caused by vitamin A deficiency (Olson, 1994a).

The estimated average requirement for vitamin A is 625 μg Retinol Activity Equivalents (RAE) for men 19 to 70+ and 500 μg RAE for women 19 to 70+ (Food and Nutrition Board, 2001). Conversion values for RAE are:

1 retinol activity equivalent (RAE)

= 1 μg of all-trans retinol
= 12 μg of dietary all-trans-beta-carotene
= 24 μg of other dietary provitamin A carotenoids

1 IU vitamin A activity

= 0.3 μg of all-trans retinol
= 3.6 μg of dietary all-trans-beta-carotene
= 7.2 μg of other dietary provitamin A carotenoids

Three kinds of vitamin A toxicity exist: acute, chronic, and teratogenic. Acute toxicity is caused by the ingestion of one very large dose of vitamin A or several large doses taken over several days (about 100 times the RDA). The effects are largely gastrointestinal upsets, headache, blurred vision, and muscle incoordination. In chronic toxicity, infants and adults show a wide range of signs and symptoms; bone and muscle pain, loss of appetite, various skin disorders, headache, hair loss, liver damage, double vision, hemorrhage, vomiting and coma. Teratogenic vitamin A toxicity causes birth defects and spontaneous abortions, and hypervitaminosis A is particularly harmful in early pregnancy (Russell, 2000).

2.2 Vitamin D

2.2.1 Source, absorption, transport, storage and excretion of vitamin D

Vitamin D is a generic term for both the pro-vitamin (pro-hormone) and the active vitamin form (Fig. 2.2). Enough sun exposure can generate sufficient active vitamin D in the

skin for the body needs. Photosynthesis of vitamin D begins with provitamin D_3 (7-dehydrocholesterol) or provitamin D_2 (ergosterol). During exposure to the sun, provitamin D is thermally isomerized to vitamin D and specifically translocated by the vitamin D binding protein into the circulation. Significant dietary sources of vitamin D include fatty fish, fortified milk, and some fortified breakfast cereals. Foods of animal origin contain vitamin D_3 (cholecalciferol), while plants contain vitamin D_2 (ergocalciferol) (Holick, 1999). Following the consumption of vitamin D-containing foods, about 80% of vitamin D is incorporated into micelles in the small intestine and then absorbed and transported to the liver by chylomicrons through the lymphatic system. Patients with chronic fat-malabsorption syndromes have trouble absorbing vitamin D and may develop a deficiency. When vitamin D enters the circulation, it is bound to vitamin D binding protein. In the liver, vitamin D is hydroxylated into 25-OH-D, the circulating form of vitamin D in the blood. Then in the kidney 25-OH-D is converted to the active hormone form of the vitamin, 1,25-$(OH)_2$ D. When there is a shortage of calcium in the blood, parathyroid hormone increases the production of 1,25-$(OH)_2$ D. The cloning of the 25-OH vitamin D-1-α-hydroxylase provided the impetus to explore the possibility that tissues other than the kidney could produce 1,25-$(OH)_2$ D. It is now recognized that many tissues in the body, including prostate, colon, skin, and osteoblasts have the capacity to express the 1-α-hydroxylase and thereby synthesize 1, 25-$(OH)_2$ D_3 (Schwartz, 1998; Cross, 2001; Tangpricha, 2001). Vitamin D is stored in adipose tissue. Eventual excretion of vitamin D metabolites takes place mostly via bile, with small amounts via the urine (Holick, 1999).

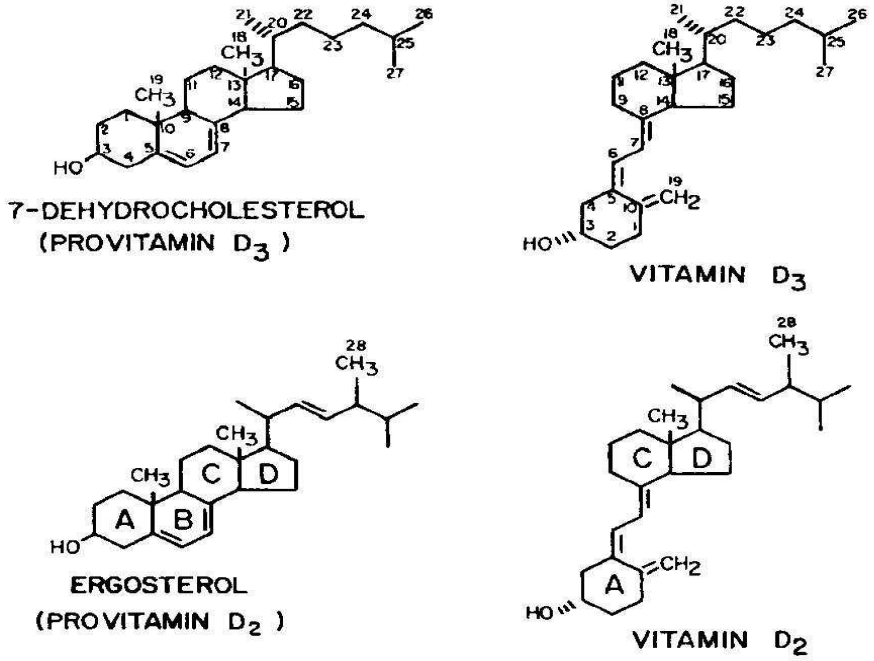

Figure 2.2 Structures of vitamin D_3 and D_2 and their respective precursors
(MacLaughlin, 1985)

2.2.2 Functions of vitamin D

The principal function of vitamin D is to maintain serum calcium and phosphorus concentration within the range that supports neuromuscular function, bone calcification, and other cellular processes. 1,25-$(OH)_2$ D activates calcium-binding protein in the small intestine, resulting in the facilitated diffusion of calcium towards the basolateral membrane. 1,25-$(OH)_2$ D also increases the absorption of phosphorus. During a time of calcium shortage, vitamin D mobilizes calcium and phosphorus from bone stores to maintain serum calcium levels. Whether vitamin D directly induces osteoblastic bone mineralization is still a matter of controversy, but the recent findings in vitamin D receptor (VDR) knockout mice have shown important clues: homozygotes of VDR knockout mice had no defects in the

development and growth before weaning, but their development was strikingly retarded after weaning. Bone formation and mineralization of the mutant mice were also severely impaired (Li, 1997; Yoshizawa, 1997). When VDR knockout mice were maintained for 50 weeks on a rescue diet containing high calcium and lactose (2% calcium and 20% lactose), they developed normally, and the impaired bone formation and mineralization were recovered completely, though they showed severe alopecia (Amling, 1999). It is likely that vitamin D is not needed for bone calcium but is responsible for maintaining extracellular calcium and phosphrus concentrations in a supersaturated state, which results in mineralization of bone.

Another function of 1,25-$(OH)_2$ D is to bind with nuclear vitamin D receptors (VDR) and regulate gene expression in target cells, resulting a variety of biological responses including proliferation and differentiation of cells. Since the 1940s, it has been recognized that people who live at higher latitudes have a higher risk of dying of the most common cancers such as colon, breast, and prostate. It has been speculated that the local production of 1,25-$(OH)_2$ D may be for the purpose of regulating cell growth, which may ultimately decrease risk of developing cancers in these tissues (Fig. 2.3; Holick, 2003). In animal studies, 1,25-$(OH)_2$ D could markedly reduce the incidence of type 1 diabetes in Non-Obese Diabetic (NOD) rats due to immune modulator effect of 1,25-$(OH)_2$ D (Hypponen, 2001). 1,25-$(OH)_2$ D is a negative regulator of the renin-angiotension system, which partly explains why African Americans who are chronically vitamin D deficient also have a higher risk of hypertension and cardiovascular disease (Harris, 2001). Recent studies also suggest that a membrane vitamin D receptor distinct from the nuclear VDR exists and is involved in events including growth, bone formation, and female reproduction (Van De Graff, 1999).

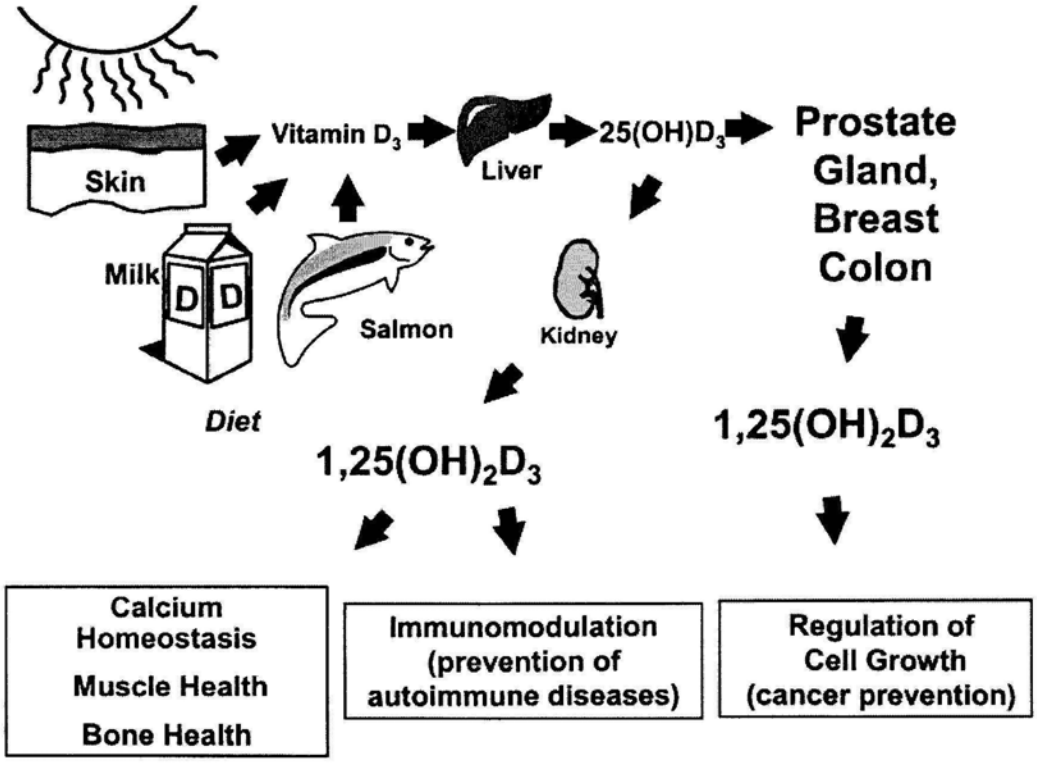

Figure 2.3 Production, metabolism and biological functions of vitamin D_3

(Holick, 2003)

2.2.3 Deficiency, toxicity and adequate intake for vitamin D

Vitamin D deficiency causes inadequate calcium and phosphorus in the blood for proper bone mineralization, resulting in bones weakening and bowing under pressure. When these occur in a child, it is called rickets; in adults, it is called osteomalacia. Signs of rickets include enlarged head, joints, and rib cage; a deformed pelvis; and bowed legs. Osteomalacia can cause fractures in the hip, spine, and other bones (Leboff, 1999).

Adequate Intake for vitamin D is 5 μg (200 IU)/day for people under age 51 and increases to 10 μg/day for people between 51-70 and 15 μg/day for older Americans. Vitamin D toxicity occurs from excess supplementation and over-fortification in milk, not

from sun exposure or food consumption. The Upper Level set for vitamin D is 50 μg /day, based on development of an excess concentration of calcium in the blood (Food and Nutrition Board, 1997). Hypervitaminosis D can lead to eventual calcium deposits in the kidneys, heart, and blood vessels. In addition, excess vitamin D can cause too much calcium to be mobilized from the bones to the blood, resulting in hypercalcemia.

2.2.4 Calcium Metabolism and Functions

Foods rich in calcium include leafy vegetables, broccoli, milk, cheese (except for cottage cheese), sardines and canned salmon. Dairy products such as milk and cheese provide about 75% of the calcium in American diets. One cup of milk contains 300 mg calcium. Skim milk is the most nutrient dense source of calcium because of its high bioavailability and low energy value (Miller, 1999). Under conditions of low calcium intake, calcium absorption occurs primarily through the transcellular mechanism regulated by vitamin D in the upper part of the small intestine (duodenum) because calcium requires a pH below 6 to stay in solution in an ionic state. By the time the acidic stomach contents reach the duodenum, they have been partially neutralized by bicarbonate released from the pancreas but are still slightly acidic. Calcium absorption within the duodenum depends greatly on the active vitamin D hormone 1,25-$(OH)_2$ D that induces the production of CaBP (calbindin), resulting in the facilitated diffusion of calcium toward the basolateral membrane. Because the intestinal contents become more alkaline as they pass down the GI tract, calcium absorption decreases at the terminal end of the small intestine and colon, although some still occurs via passive absorption, the paracellular, concentration-dependent diffusional process that takes place throughout the length of the intestine. In addition, with high calcium intake, most of the

calcium will be absorbed by the paracellular process, largely in the distal portions of small intestine, because of the relatively large mass of calcium and down-regulated vitamin D-dependent transcellular process (Bronner, 2003). When the body needs extra calcium such as during infancy and pregnancy, calcium absorption rate might rise from 25% to 60% (Food and Nutrition Board, 1997). All components of the diet that make calcium soluble or keep it in solution within the ileum should stimulate passive diffusion. Several molecules, particularly milk proteins like the phosphopeptide derived from casein, and amino acids like L-lysine and L-arginine can form soluble chelates with calcium and enhance the absorption (Gueguen, 2000). Animal studies produced strong evidence that lactose has beneficial effects on intestinal calcium absorption and calcium retention in bone, though results from human studies are inconclusive (Zittermann, 2000).

Normal blood calcium is maintained by multiple hormonal systems, which makes blood calcium a poor measure of calcium status. When blood calcium falls, the parathyroid gland releases parathyroid hormone (PTH), which works with 1, 25- $(OH)_2$ D increasing the kidney's retrieval of calcium before it is excreted in the urine. PTH also indirectly increases calcium absorption by increasing the synthesis of 1, 25- $(OH)_2$ D. In addition, PTH with 1, 25- $(OH)_2$ D causes calcium release from bones by stimulating the activity of osteoclasts. When blood calcium is too high, the release of parathyroid hormone falls, resulting in increased loss of calcium from kidneys and decreased bone absorption. The thyroid gland secretes calcitonin, which decreases calcium loss from bones (Van de Graff, 1999). Other routes for calcium loss include the skin and the feces.

Calcium plays major roles in bone development and maintenance. In addition, calcium ions participate in several reactions in the cascades that lead to the formation of fibrin, the

main protein component of a blood clot. Further, calcium plays critical roles in muscle contraction and transmission of nerve impulses to targeted cells. Calcium ions also help regulate cell metabolism by participating in the calmodulin system. Calcium was suggested to be a key factor for VDR transcription (Zineb, 1998): when vitamin D deficiency decreased VDR mRNA in skin keratinocytes, kidney, and duodenum of adult rats, only the calcium-lactose diet increased duodenal VDR mRNA while vitamin D supplement could not. Other possible functions of calcium include lowering blood pressure in some people, reducing the risk of colon cancer by binding bile acids and free fatty acids in the lumen of the colon, and lowering blood cholesterol, etc.

The most common sign of calcium deficiency is osteoporosis, which leads to approximately 1.5 million fractures per year. Regular sun exposure and consumption of food sources of calcium and vitamin D are important for bone health. The adequate intake for calcium for adults ranges from 1000-1200 mg/day. For adolescents between the ages of 8 and 18, the AI is set at 1300 mg/day to allow increases in bone mass during growth and development. Calcium in food does not pose a health threat because it is present in relatively modest amounts, but excessive amount of supplement will cause calcification of kidneys and other organs. The Upper Level for calcium is 2500 mg/day, based on the risk of developing kidney stones (Wardlaw, 2002).

2.3 Fortification of vitamins A and D in milk

2.3.1 History of Vitamins A and D fortification

In the late 1930s vitamin and mineral deficiency were prevalent in the U.S. and the public health need for food fortification was evident. Rickets was a common disorder of

children with an estimated 75% of infants in New York City afflicted in 1921 (Backstrand, 2002). To assist in reducing the prevalence of rickets, AMA's Council on Foods and Nutrition recommended vitamin D fortification of fluid milk products in the U.S. (Stevenson, 1955). Since the fortification of this nutrient, rickets has almost been eradicated. The wide acceptance of milk fortification with vitamin D led to consideration of the addition of other nutrients. Vitamin A fortification was initiated in the 1940s when physicians occasionally encountered night-blindness; fortification of margarine was reported practiced even before the structure of this vitamin was determined. In response to high rates of malnutrition and fears of potential involvement in war, President Roosevelt called a National Nutrition Conference for Defense, which met in May of 1941. At this meeting, the Committee on Food and Nutrition of the National Research Council (now the Food and Nutrition Board) presented the first RDAs, which serves as the yardstick for fortification (NRC, 1943).

2.3.2 New Fortification policies

FDA 1980 guidelines endorse the addition of nutrients to foods under four conditions: nutritional deficiency; restoration of nutrient losses; improving the quality of a replacement food; balancing nutrient content of food. Unfortified whole milk is considered a good source of vitamin A, which is associated with the fat fraction; levels range from 400 IU in winter to 1200 IU in summer. As reduced fat milks became popular in the U.S., the reduction of vitamin A in these products became a nutritional concern. Therefore, the Pasteurized Milk Ordinance (PMO) (PHS/FDA, 1999) states that reduced fat milk products must be fortified with vitamin A to the nutritional equivalence of the general milk standard. Currently, low fat and skim milks are required to be fortified to contain vitamin A at not less than 2000 IU/qt.

Although not required, vitamin A could be added to whole milk at 2000 IU/qt. In contrast, whole milk is not considered a good source of vitamin D, with levels ranging from 5 IU/qt in winter to 40 IU/qt in summer (Kurmann, 1994). So removal of fat is not considered to render milk inferior for vitamin D. Therefore, fortification of vitamin D is optional for all milks. However, if added, vitamin D must be present at 40 IU per quart. About 98% of milk marketed in the U.S. is fortified with vitamin D.

2.3.3 Current needs for vitamins A and D fortification

Vitamin A is essential for child health and survival, and vitamin A deficiency is now recognized as a major contributor to child mortality in developing countries. Americans are at little risk of vitamin A deficiency, since the vitamin is abundant in the food supply. But there is a high frequency of vitamin A deficiency in patients with Crohn's disease and other chronic gastrointestinal diseases. Foods fortified with vitamin A include cooking oils, margarine, milk, sugar, and ready-to-eat cereals. In Costa Rica, fortification of sugar was practiced from 1977 to 1981, and the prevalence of vitamin A deficiency in children dropped from 33% in 1965 to 2% in 1980 and rose to 9% in 1996, leading to a renewed interest to resume sugar fortification in the country (Mora, 2000).

Though rickets has been thought cured, during the last 5-8 years in particular, resurgence of rickets has been described (Abrams, 2002). Increasing case reports are related to low dietary intake of vitamin D and calcium, and decreased sunshine exposure. More than half of the 30 cases of rickets in NC between 1990 and mid-1999 occurred during the last 18 months of the surveillance period. Rickets is also making resurgence in African American young children who receive their total nutrition from breastfeeding (Kreiter, 2000). What is not

appreciated is that vitamin D deficiency is common in otherwise healthy, young, middle aged, and older adults (Outila, 2001). A recent survey of women of child bearing age in the United States revealed that at the end of the winter 41% of African American women aged 15-49 years were found to be vitamin D deficient, compared with 4% of Caucasian women at the end of summer (Nesby-O'Dell, 2002). Given an increasing need for vitamin D fortification, NAS points out that estimates of vitamin D intake are complicated by the fact that there are contributions from food (which are difficult to estimate) as well as contributions from subcutaneous production of vitamin D_3 with exposure to sunlight. In 2000, FDA denied a petition to require the addition of calcium and vitamin D rather than continue the optional addition of these nutrients (FDA, 2000).

2.3.4 Fortification compliance

The FDA mandated that vitamin concentration for fortified fluid products must be within 100 to 150% of the label claims (FDA, 1999), in contrast to the previously accepted 80 to 120%. So the acceptable range for vitamin D is 400-600 IU/qt, and 2000-3000 IU/qt for vitamin A. And each manufacturer must have representative milk products assayed at least annually in a laboratory certified by the FDA with methods that are acceptable to the FDA (Nichols, 1992). Multiple studies found that a high percentage of fortified milk products failed to meet label claims: most of the milk out of compliance was under-fortified (Holick, 1992; Nichols, 1992). From January 1997 to December 2000, compliance of fluid milk fortification in NY State was studied in Cornell University (Murphy, 2001). Data showed that only 44.5% and 47.7% of the milks tested in the study were within the acceptable range for vitamins A and D, respectively. Most samples out of compliance were below acceptable

limits, with 51.4% below 2000 IU/qt vitamin A and 46.3% below 400 IU/qt vitamin D. And samples with values below 1000 IU for vitamin A (12.8%) and 160 IU for vitamin D (12.0%) suggest significant shortcomings in fortification protocols. Skim milk tends to be under-fortified, but vitamin D concentrations did not appear to vary by milk type. On the other hand, over-fortification will cause toxicity. In 1992, over-fortification of vitamin D in milk from a small in-state Boston plant caused toxicity in 8 patients and the vitamin D levels were reported 70-600 times above the amount stated on the label (Jacobus, 1992). This is the first reported case of hypervitaminosis D from a commercial food product in the U.S. since fortification became common in this country in the early 1930s. In contrast to vitamin D, there are no reports of vitamin A toxicity attributed to the intake of fortified foods, even in developed countries where more than one food is fortified. An intake of 10,000 IU/day has been set as the level safe for adults, including pregnant women. Enormously high and practically impossible amounts of an individual food (except for liver) or a combination would have to be consumed on a daily or regular basis to reach this toxicity. However, in theory, the potential risk of toxicity may rise, as an increasing number of foods are fortified, especially when the food vehicles are staples.

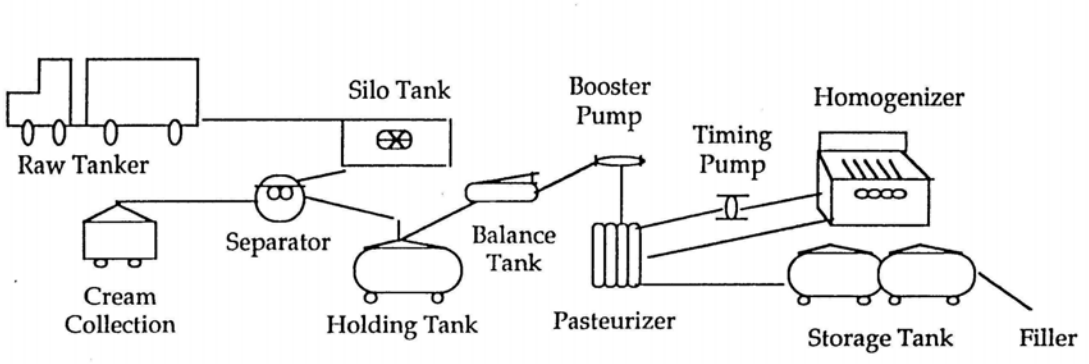

Figure 2.4 Schematic graph of typical dairy plant processing

2.3.5 Factors that may contribute to improper fortification

1. Fortification points (Fig. 2.4)

Adding vitamin preparations before fat standardization and separation might cause errors in fortification level due to vitamin extraction with the fat fraction and vitamin loss caused by settling at the bottom due to incomplete mixing. According to the Pasteurized Milk Ordinance (PMO), the addition of vitamins must be accomplished prior to pasteurization. Vitamin preparation addition just prior to pasteurization seems to be the optimal choice (Hicks, 1998).

2. Metering pump vs. batch addition

Metered injection usually is done after separation and prior to homogenization and calibration checks are based on the product flow rate; it is used with in-line fat standardization, which allows switching without stopping products. Batch addition uses only accurate, calibrated measuring devices, such as pipettes or syringes. Accurate measurement of milk volume, vitamin concentrates, and sufficient mixing time is required, ideally after the milk is standardized. Fortification variation was not dependent upon the method of addition (PHS/FDA, 1999).

3. Fortification carriers

Carriers can be butter oil, corn oil, evaporated milk, non-fat dry milk, polysorbate 80, propylene glycol and glycerol mono-oleate. In general, oil-based vitamin A preparations increased the stability as the fat content of the milk samples increased, but added vitamin A was more stable in a skim milk system when water-based carriers were used (Fellman et al, 1991). However, vitamin degradation can occur before the addition of the vitamin concentration to milk. Processors may leave the vitamin preparations at room temperature for

long periods of time or repeatedly open and close the same container, which may expose preparations to excessive amounts of light, heat and oxygen. It is best to store all concentrates under refrigeration unless manufacturer's directions indicate otherwise.

4. Stability of vitamins A and D in milk stored in different containers

Milk containers can be paperboard, glass, and polyethylene, and a few other materials. A study conducted by Paredes (1996) indicated that the maximum sorption of vitamin A was 63% and of vitamin D was 58% by low-density polyethylene (LDPE) plastic bottle and up to 55% for vitamin A and 47% for vitamin D sorption by high-density polyethylene HDPE plastic. The sorption occurred within one hour of contact with fortified skim milk. So skim milk may not meet requirements for vitamins A and D if packaged in polymeric materials. Vitamins are sensitive to light, heat and oxygen. Vitamin D is very stable in homogenized whole milk and there is no loss of vitamin D in fortified homogenized whole milk during long periods of proper storage. On exposure to light, there was slight loss of vitamin D_3 from fortified milk. Air exposure did not affect stability (Renken, 1993). However, vitamin A and D-fortified reduced fat milks are subject to decrease in vitamins A and D (Tanner, 1988). Both natural and artificial light, particularly with wavelengths ranging from 420-520 nm can induce vitamin A degradation and flavor defects. Senyk and Shipe (1981) demonstrated a reduction in vitamin A content of whole, reduced fat and skim milk exposed to 2000 lx for 4 h. The presence of milk fat appears to protect against vitamin A degradation in milk, but adversely affects the flavor quality after exposure to light (Gaylord, 1986 and Whited, 2002). A brief, moderate light exposure (2h, 2000 lx) can reduce the nutritional value and flavor quality of fluid milk. Furthermore, packaging type and size influences vitamin A loss and reduction of flavor quality. Of milk packaged in polyethylene terephthalate (PET), PET with

a UV blocker, low-density polyethylene (LDPE) or high-density polyethylene (HDPE), products in HDPE had the largest vitamin A losses (Cladman, 1998 and van Aardt, 2001). The majority of US fluid milk products currently are packaged in containers made of paperboard or HDPE plastic. Paperboard containers that are usually coated with LDPE offer protections from light exposure, but HDPE containers do not. Paperboard may shield milk from about 98% of the light, while plastic containers only provide 30% protection; glass containers only provide 9% (Fellman et al, 1991).

2.4 Beta-lactoglobulin (BLG)

2.4.1 What is BLG?

Beta-lactoglobulin (BLG) is a major whey protein in bovine milk composing 7 to 12% of skim milk total proteins (Perez, 1990). BLG was first isolated 60 years ago and a wealth of chemical and biological information has been accumulated. BLG is a globular protein with a molecular weight of about 18,000. The crystal structure shows that the core of the molecule is made up of a very short α-helix segment and eight strands of anti-parallel β-sheet, which wrap around to form an anti-parallel β-barrel (Fig. 2.5) (Papiz et al, 1986). The secondary structure of bovine BLG is 15% of α-helix, 50% of β-sheet, and 15-20% of reverse turn (Creamer et al, 1983). BLG is acid stable, resisting denaturation at pH 2.0. BLG generally exists as dimers, resulting from the association of monomers, and the dimers begin to dissociate at pH above 5.6. At pH 8.6 and above, BLG undergoes a polymerization that progresses with time and that seems mainly due to oxidation of the sulfhydryl groups. BLG is quite resistant to hydrolysis in vitro by proteases such as trypsin, chymotrysin and pepsin (Reddy et al., 1988). Similarly,

BLG seems to be quite resistant to gastric digestion in vivo and remains mostly intact after it passes through the stomach or calf abomasum (Yvon et al., 1984). At least one study suggests absorption of some intact BLG in humans, as this protein can be detected in trace amounts in maternal milk from women who have consumed cow's milk (Monti et al., 1989). Bovine BLG denatures above 65°C at pH 6.7, followed by aggregation. The protein is most heat sensitive near pH 4.0, with a maximum stability at pH 6.0 and decreasing stability in the higher pH range (Relkin and Launay, 1990).

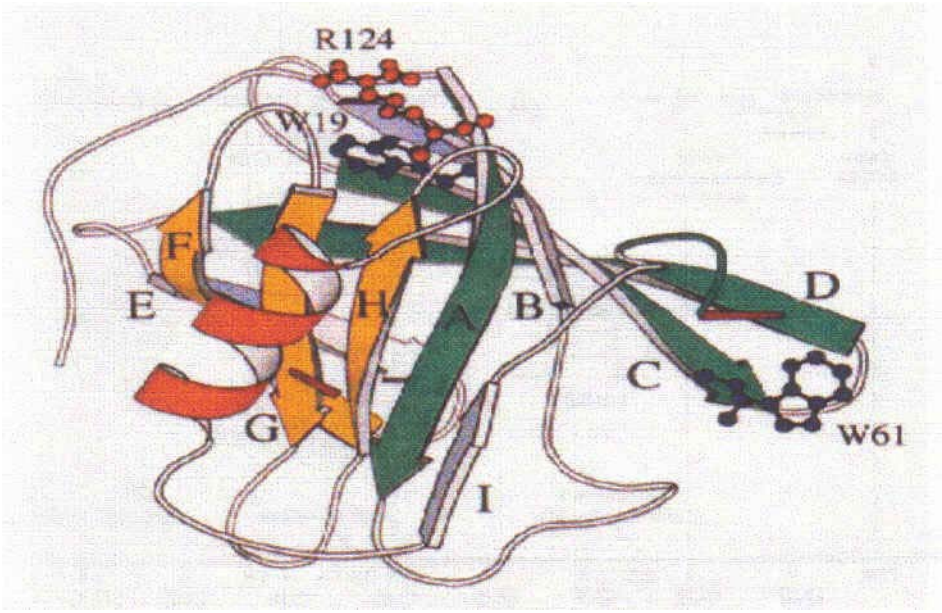

Figure 2.5 A representation of bovine beta-lactoglobulin

red, α-helix; yellow and green, β-sheets; purple, disulfide bonds. The location of Trp19, Trp 61 and Arg 124 is indicated (Brownlow, 1997).

2.4.2 Functions of BLG

The physiological functions of BLG remain elusive. However, because of its binding affinity for retinol (Wu, 1999 and Wang, 1997) and fat-soluble molecules (Frapin, et al., 1993), BLG is thought to function in retinol transport and enhancement of pregastric esterase

activity by binding of inhibitory fatty acids (Perez, et al., 1991). BLG is in the family termed lipocalins, a class of small globular proteins that interact with hydrophobic molecules. Retinol binding protein (RBP) and apolipoprotein D also belong to the same family and BLG was reported to share similar amino acid sequence and tertiary structure with RBP. It may play a role in the delivery of retinol to the intestinal absorptive cells after hydrolysis of esterified retinol in the lumen. The effect of bovine milk BLG on intestinal uptake of retinol was examined in suckling rats with the everted gut-sac technique (Said et al, 1989). Uptake of retinol bound to β-lactoglobulin was significantly higher than that of free retinol both in the jejunum and the ileum. The enhancing effect of BLG on retinol uptake was specific because equimolar concentrations of bovine serum albumin and lactoferrin had no effect on retinol uptake. These results demonstrate that β-lactoglobulin specifically enhances intestinal uptake of retinol and suggest the possibility of a receptor for β-lactoglobulin-like proteins at the brush border membrane of the enterocyte (Said et al., 1989). It is important to note that the β-lactoglobulin/RBP-assisted absorption proposed is a completely separate pathway for entry into intestinal cells from the micellar mechanism of free retinol. Therefore, fortifying milk with a form of vitamin A that could use both of these pathways should increase the rate of vitamin A absorption when vitamin A status is poor.

2.4.3 Interaction of β-Lactoglobulin with retinyl palmitate and vitamin D_3

BLG has two hydrophobic pockets that are potentially capable of binding lipophillic molecules: one in the calyx formed by the β-barrel and the other between the α-helix and the surface of the barrel. Although binding sites for ligands remain controversial, most evidence indicates that retinoids bind in the calyx and palmitate binds near the monomer contact surface

in the dimer (Wang, 1999). Binding of retinol by BLG quenches the fluorescence of Trp19 of the protein, which lies at the bottom of the calyx (Fig. 2.5); while interaction of BLG with palmitate increases the tryptophanyl fluorescence; therefore fluorescence changes can be used to characterize the binding of both retinoids and fatty acids. Fluorescence spectroscopy suggests that BLG is capable of binding two moles of retinyl palmitate per mole of protein. However, recent crystallographic evidence (Wu, 1999 and Kontopidis, 2002) suggests that retinol and palmitate competitively bind to the same position, which is the central calyx, and the latter displacing the former. Similar analyses of fluorescence changes occur when BLG is titrated with vitamin D_3 and it seems two molecules of vitamin D_3 bind with both sites on one molecule of the protein (Wang, 1997).

2.5 Assays of vitamins A and D by HPLC

2.5.1 Vitamin A sample preparation

Retinoids are very sensitive to light (wavelengths below 500 nm), oxygen, trace metals, strong acids, and excessive heat. All sample treatment should be done in amber vials under yellow or red light. Antioxidants like BHT and nitrogen purging can be beneficial for long-term storage at -20°C or -80°C. The use of an internal standard usually strengthens a method and adds better precision and accuracy to a quantitative assay. Retinyl acetate is a commonly used internal standard due to its absence in most natural samples, co-elution with the analyte, and a functional group common to other retinoids (Gundersen, 2001). If an extraction procedure includes saponification (alkaline digestion), retinyl acetate cannot be used. However, adding it volumetrically after saponification or prior to injection is helpful. Homogenization is often needed for non-fluid samples. Two to four volumes of water

miscible organic solvents such as ethanol are necessary for protein precipitation when there is no further clean-up procedure. Residual proteins will lead to backpressure and deterioration of the column. Extraction by non-polar solvents such as hexane is usually repeated three times to improve the recovery (Sharpless, 1995). To release retinoids from a complex matrix and to hydrolyze retinyl conjugates, saponification of samples is necessary. However, this method provides no information on the amount of unesterified retinol or the nature of conjugation (esters or glucuronides) in the sample. Saponification is generally performed under heat with the use of 5% ethanolic potassium hydroxide containing pyrogallol at 60 °C for 20 min (Ross, 1986). After cooling, retinol is extracted with hexane or diethyl ether. At extreme temperatures, cis-trans-isomerization cannot be excluded.

2.5.2 Vitamin D sample preparation

The use of an internal standard technique provides higher analytical confidence. Since vitamin D_3 is fortified in dairy foods and present naturally in animal samples, vitamin D_2 is an ideal choice of internal standard given the virtually identical physiochemical properties: recovery, λ_{max} and extinction coefficient. Saponification is obligatory for determination of very low concentrations of vitamin D in fatty foods and samples. Hot saponification results in the thermal isomerization of vitamin D to previtamin D. Thompson et al. (1977) reported that saponification of milk at 83 °C in the presence of pyrogallol results in a 10-20% loss of added vitamin D due to thermal isomerization. To remove the bulk of the lipid components in the samples, the relatively mild saponification procedure performed by flushing with nitrogen at ambient temperature and overnight (Indyk and Woolard, 1985) is recommended. Higher recovery and less thermal isomerization can be achieved. Ethanol is often used to precipitate

protein before multiple extractions by an organic solvent such as hexane. The organic layers are then combined and washed with water or ethanol/water several times to reach neutral pH. Though solid phase extraction by a silica cartridge has been used as a clean-up procedure for removal of interfering artifacts by many researchers, it is optional for vitamins A and D fortified skim milk due to relatively low levels of endogenous α-tocopherol, carotenoids, sterols, and other interfering substances (Muniz, 1982). The extract is evaporated to dryness under nitrogen and re-dissolved in the HPLC mobile phase for analysis.

2.5.3 HPLC methodology

High-performance liquid chromatography (HPLC) is a predominant method for separation and quantification of retinoids and vitamin D in biological samples. HPLC is divided into normal-phase and reversed-phase (RP): in normal-phase HPLC the stationary phase (the column) is polar such as silica or silica modified with CN or NH_2 groups. The mobile phase is non-polar solvents such as hexane with a small amount of more polar solvent such as iso-propanol or chloroform. In the RP-HPLC the column is made of non-polar material, typically C18 or C8, and the mobile phase is more polar. Normal phase HPLC is very sensitive to small changes in mobile phase composition, resulting in shifts of retention time; however, it is often applied to separate cis/trans isomers of retinoids (Barua, 2000). A major practical advantage of normal-phase HPLC is that there is no need to evaporate extracts to dryness and vitamin A is maintained in its relatively stable ester form throughout the assay and is protected by the lipids to the point of chromatographic separation (Thompson, 1980). In contrast, RP-HPLC is more stable, can simultaneously analyze polar and non-polar retinoids, and is able to resolve different ester forms of retinol. A much longer

time is needed for RP-HPLC analysis and re-equilibration of the column because of the solvent gradient (Lanvers, 1996). It is necessary to evaporate the extract (in a non-polar solvent) to dryness and to dissolve the residue in a small amount of methanol or the mobile phase.

Most published procedures for simultaneous analysis of retinol and retinyl palmitate utilize RP-HPLC and a solvent gradient, with the elution time for the retinyl palmitate peak ranging from 15 min to 40 min. Cohn (1988) and Krasinski (1990) reported using normal-phase HPLC with a two-solvent gradient system. Ruotolo (1992) described a most simple and efficient protocol for studying the kinetics of retinyl esters in whole plasma and several lipoprotein fractions following the consumption of an oral fat load containing retinol. The author directly applied the lipid fraction onto the silica 5 μm normal-phase column, and retinyl palmitate, retinyl acetate and retinol were eluted separately within 10 min by the mobile phase prepared from hexane: n-butyl chloride: acetonitrile (82:13:5 by volume, with 0.1 ml acetic acid). This method greatly shortened the analysis time and efficiently resolved retinol and retinyl acetate without using a solvent gradient in a normal-phase system.

HPLC is believed to offer more rapid analysis and efficient separation than conventional vitamin D assay methods. The AOAC official liquid chromatographic methods determine total and preformed vitamin D but do not discriminate vitamin D_2 and D_3 using cleanup and analytical silica columns (AOAC, 1995). The inability of normal-phase HPLC to resolve vitamins D_2 and D_3 means that one vitamer cannot be used as an internal standard for the other (Jones, 1975). C-18 Vydac 201TP reversed-phase columns have been reported to achieve good separation of vitamins D_3 and D_2 and reproducible retention (Villalobos, 1990 and Sliva, 1992). Mobile phases such as acetonitrile: ethyl acetate: chloroform (88:8:4) and

methanol: water (98:2) have been used for C-18 Vydac and Octadecyl Silane (ODS), respectively. A modified HPLC method with a linear gradient program was described by the Milk Quality Improvement Program Laboratory at Cornell University (Murphy, 2001). In this system, vitamins D_2 and D_3 were well eluted from the 4.6 × 250-mm Vydac TP201 C18, 5 μm column by the mobile phase acetonitrile: ethyl acetate: chloroform (88:8:4) within 15 minutes. Methanol flushing after each sample run helped remove polar impurities attached to the column and improved column performance. The assay time for one sample is 40 min including column conditioning and equilibration. Normal-phase/reversed-phase chromatography is the ideal combination for semi-preparative and quantitative separations in two-dimensional HPLC. Vitamins D_2 and D_3 co-elute during the semi-preparative stage, allowing a narrow retention window to be collected for analysis using internal standardization (Nollet, 2000). By this means, Johnsson, et al (1989) obtained a vitamin D_3 detection limit of 0.1 μg/kg for milk and milk products.

UV-VIS absorption detector is very popular for HPLC because of its ease of use, broad application area, and low price. The detector measures the change in absorbance of light in the 190-350 nm region (UV) or the 350-700 nm region (visible). This type of detector allows operation at the wavelength of maximum absorption of the analyte or at a wavelength that provides optimum selectivity. Another photometric detector often used is the Photodiode Array (PDA) detector, which passes the total light through the flow cell and disperses it with a diffraction grating. The dispersed light is measured by an array of photosensitive diodes. PDA detectors can simultaneously measure the absorbance at all wavelengths versus time. The selectivity of absorbance measurement for a given vitamin is dependent upon the wavelength employed in the measurement and the strength of absorbance of the vitamin

relative to the absorbance of interfering substances. Most lipids found in foods do not have chromophores that absorb strongly in the UV region above 220 nm except for free or esterified conjugated fatty acids with λ_{max} of 230-235 nm for dienes and 260-280 nm for trienes. Glycerides and sterols exhibit weak, but measurable, UV absorbance within the spectral range of vitamin D. The absorption spectra of vitamin A lie beyond those for glycerides and sterols. Also, for these photometric detectors a careful selection of solvents utilized as mobile phases is required based on solvents' UV cutoffs. Other types of detectors include fluorescence and electrochemical detectors (Nollet, 2000).

Chapter 3 Preparation of protein-vitamin complexes

3.1 Protein-vitamin binding study

3.1.1 Introduction

As a member of the lipocalins family, BLG is capable of binding fat-soluble compounds, including vitamin A palmitate and vitamin D_3 (Wang, 1997 and Wu, 1999). Therefore, these hydrophobic molecules might be sheltered against oxidation in complexes with the protein. BLG is proposed to be a potential carrier for vitamins in reduced fat dairy products. Oxidation of anhydrous milk fat was reported significantly limited by micro-encapsulation in WPI (Moreau, 1996). The purpose of this experiment was to verify the capacity of BLG in the Bio-Pure BLG (Davisco) to bind vitamins A and D. Evidence from crystallography and fluorescence spectroscopy suggests that the putative binding site is the central calyx formed by the eight-stranded antiparallel β-barrel of the protein. The fluorescence emission of Trp 19 at the bottom of the calyx is quenched at 332 nm when a ligand is bound (Dufour, 1990), which was used to characterize the binding of vitamins A and D.

3.1.2 Materials and methods

Bio-Pure BLG was provided by Davisco Foods International, Inc. (Le Sueur, MN). Pure retinyl palmitate, retinyl acetate, vitamins D_3 and D_2 were purchased from Sigma Chemical Co. (St. Louis, MO). All the other chemicals and reagents were purchased from Sigma (St. Louis, MO) or Fisher (Pittsburgh, PA), and were of the highest analytical quality. Equipment included balance (MCI Analytic AC 210S, Sartorius, Inc. Edgewood, NY), Genie Vortex mixer (Fisher Scientific, Raleigh, NC), and micropippetors (Eppendorf and Oxford).

Before the preparation of protein-vitamin complexes, the electrophoresis was performed on an SDS-PAGE apparatus (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) to test the purity of the protein from Davisco. Data suggested beta-lactoglobulin was approximately 90% of the total protein, which was consistent with that in the product information (data not shown). The protein was dissolved in 50 mM phosphate buffer (pH 7.0) to give a 0.5 mM stock solution. Concentrations of BLG in solution could be determined on the Genesys 2 spectrometer (Spectronic, Thermo Electron Scientific Instruments Co. Madison, WI) by using a molecular absorption coefficient $\in_{278}$ = 17600. Vitamin D_2 and D_3 stock solutions were made 200 μg/ml in methanol. Retinyl palmitate and retinyl acetate stock solutions were made 200 μg/ml in ethanol. The UV absorption (A) of all vitamin working solutions (1:100 dilution) was measured on a spectrometer at 264 nm for vitamin D and 325 nm for vitamin A, and the formula C (g/100ml) = A/ $E^{1\%}$ was used to calculate the exact concentration of the vitamin working solutions. The absorption coefficients E (1%/1cm) are 460 and 485 for D_2 and D_3, and 940 and 1560 for retinyl palmitate and retinyl acetate, respectively. Absorption spectra of the solutions were also compared with standard absorption spectra: decrease in peak absorption or change in pattern of the absorption spectra indicated degradation of vitamins in the solutions.

3.1.3 Results of fluorescence spectroscopy

Fluorescence spectra were performed on a System 3 Scanning Spectrofluorometer (Optical Technology Devices, Inc., Elmsford, NY) to verify binding of the protein with vitamins A palmitate and vitamin D_3 (Dufour, 1990 and 1991). Fluorescence emission spectrum of 10 μM β-lactoglobulin in 50 mM phosphate buffer (pH 7.0) was measured in the

absence and in the presence of 10 μM retinyl palmitate dissolved in ethanol. A solution of N-acetyl-tryptophanamide that had an absorbance at 287 nm, which was equal to that of the protein solution, was titrated in a similar manner to serve as a blank sample. The measurement was performed at 21°C using an excitation wavelength of 280 nm and a scan speed of 50 nm/min. The fluorescence intensity enhancement at 284 nm and fluorescence quenching at 332 nm are quantitatively related to retinyl palmitate binding (Fig.3.1).

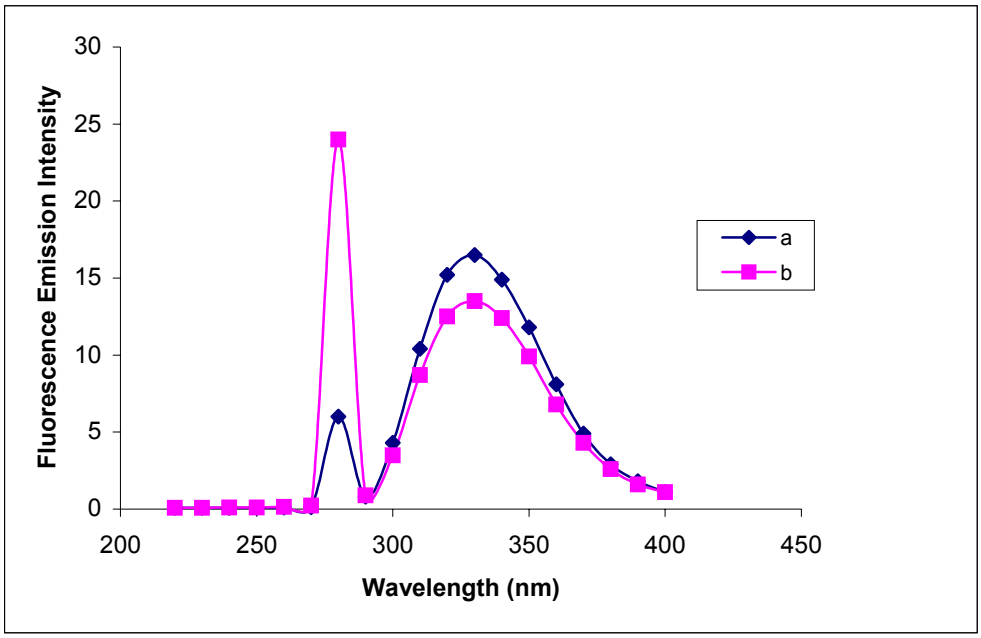

Figure 3.1 Fluorescence emission spectrum of BLG in the absence (a) and presence (b) of retinyl palmitate

Fluorescence emission spectrum of 1 μM β-lactoglobulin in 50 mM phosphate buffer, pH 7.0, was also measured in the absence and in the presence of 2 μM vitamin D_3. The measurement was performed at 21°C using an excitation wavelength of 287 nm and a scan speed of 50 nm/min. The fluorescence intensity quenching at 332 nm is quantitatively related to vitamin D_3 binding (Fig.3.2).

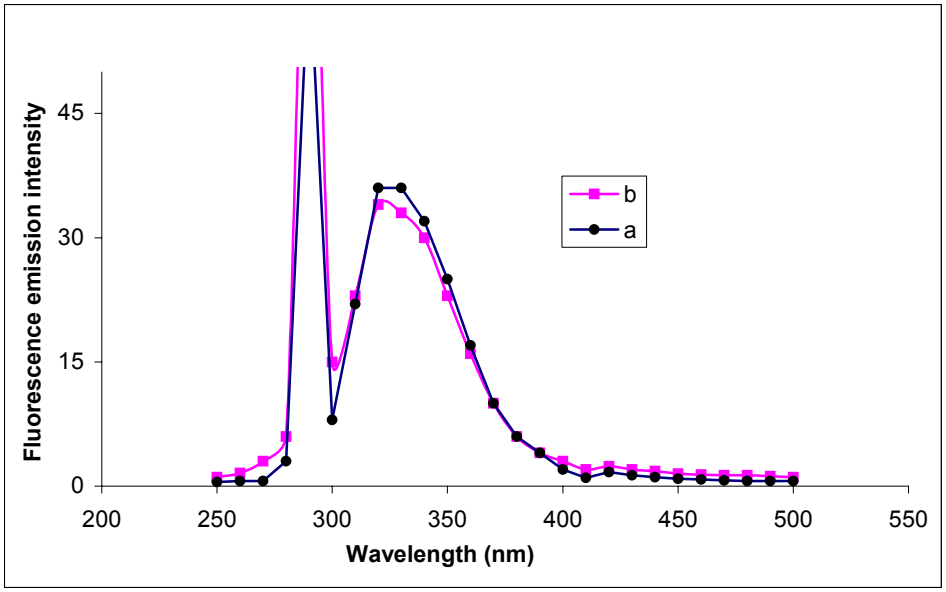

Figure 3.2 Fluorescence emission spectrum of BLG in the absence (a) and presence (b) of vitamin D_3

3.1.4 Discussion and Conclusion

Binding of vitamins A and D with the BioPure BLG was confirmed by quenching of the protein fluorescence at wavelength 332 nm. The fluorescence emission spectra were similar to those of pure BLG binding with vitamins (Wang, 1999), which suggested that the BioPure BLG from Davisco is appropriate for use in preparing BLG-vitamin complexes as vitamin fortifiers. Data collected were not sufficient for calculating apparent dissociation constants and binding stoichiometry for binding of vitamins to BLG. As putative evidence suggests that BLG is capable of binding at least 1 mole of vitamin A palmitate or vitamin D per mole (Wang, 1997 and Wu, 1999), 1:1 molar ratio of pure protein and vitamins were used when preparing protein-vitamin complexes.

3.2 Spray-drying of BLG-vitamin complexes

3.2.1 Introduction

Spray drying is the most used drying method in the food industry. In a typical spray dryer, a solution, suspension, or emulsion is pumped to an atomizer (a rotating wheel or a nozzle), which sprays the liquid into a high-velocity stream of hot air, producing droplets. As the droplets pass through the hot air flow that can be co-current with the liquid, counter-current, or a combination of both, the moisture rapidly evaporates. The large particles fall to the bottom of the chamber and are collected. Fine particles entrained with the exhaust air are generally collected by passing the air through a series of external cyclones, scrubber, or bag filters. The heat and mass transfer during drying occurs in the air and vapor films surrounding the droplet. This protective envelope of vapor keeps the particle at the saturation temperature. As long as the particle does not become completely dry, evaporation still takes place and the temperature of the solids does not approach the dryer outlet temperature. As a result, heat-sensitive products can be spray dried at relatively high air temperature (Mermelstein, 2001). Whey protein isolate (WPI, mainly BLG) has been successfully used as an efficient encapsulation agent by spray drying (Moreau, 1996). The purpose of this experiment was to spray-dry the BLG-vitamin complex solutions and produce stable and convenient fortifiers of vitamins for shelf life studies.

3.2.2 Materials and Methods

To a solution of 200 ml 15% BLG (BioPURE, Davisco) in water, pure retinyl palmitate or vitamin D_3 (Sigma Chemical Co., St. Louis, MO) was slowly added at a 1:1 (protein/vitamin, mol/mol) ratio. Since fat-soluble vitamins are not miscible with water, vitamins were pre-

dissolved in ethanol (<3% final concentration) and allowed to incubate 30 min after addition into the protein solution. The spray dryer (Anhydro Co., Tonawanda, NY) was preheated and completely dried until the inlet temperature reached 130°C and the exhaust temperature approached 80°C (the regulator of the electrical heater was set to ~3KW). The air pressure was set at 15 psi. The chamber door was tightly closed and the flap valve above the sample bucket was set open while spray drying. De-ionized (DI) water was fed through for 10 min to allow stabilization of the system. The protein-vitamin solution was then pumped using a MasterFlex peristaltic pump (Model 7518-10, Cole-Parmer Instrument Co., Vernon Hills, IL) at a constant flow rate of 15 ml/min (0.5 on the speed scale). After the spray-dried powder was carefully recovered from the sample bucket, hot soapy water and DI water were fed to rinse the sample feeding pipe and nipple. The chamber, cyclone and powder duct were rinsed with hot tap water and allowed to air-dry.

3.2.3 Recovery of protein and vitamin from spray drying

Vitamin A palmitate 0.5 g and vitamin D_3 0.5 g (dissolved in 0.75 ml ethanol) were separately spiked into 200 ml 15% BioPURE BLG water solution. After spray drying, 21 g of protein were recovered from the original 30 g. Recovery of the protein was 70%. For HPLC analysis, the spray-dried powder was accurately weighed and dissolved in DI water, and the vitamins in the spray-dried powder was extracted and assayed according to the procedures addressed in section 4.2. Retinyl acetate and vitamin D_2 were added as internal standards, respectively. The retention times are 3.76 min and 6.37 min for retinyl palmitate and retinyl acetate, and 11.15 min and 13.03 min for vitamin D_2 and D_3 are, respectively (Figures 3.3 and 3.4). Based on the average AUC ratios of RP/RA and D_3/ D_2, the average

level of retinyl palmitate was 2.88 μg per 50 mg powder and vitamin D_3 level was 3.65 μg per 50 mg powder as calculated from the standard curves.

3.2.4 Discussion and conclusion

The recovery rate for vitamins was very low compared to that of the protein. Possible reasons include: 1. Ethanol denatured the protein and decreased protein binding with vitamins. 2. High temperature denatured the protein and negatively affected the binding. 3. Because fat-soluble vitamins are not water dispersible, the interaction of protein and vitamin was not efficient in water solution in the absence of emulsifiers. 4. Whey protein in the solution may have been high enough that the BLG formed dimers, which would affect the binding of the protein with vitamins.

HPLC chromatograms (Fig 3.3 and Fig 3.4) showed a few *cis*-isomer peaks for retinyl palmitate together with a dominant all-trans isomer peak, indicating vitamin A isomerization occurred during the spray drying procedure. For future research, exhaust temperature lower than 65°C is recommended, given that beta-lactoglobulin denatures above this temperature and vitamin isomerization increases at higher temperature. The use of ethanol to dissolve fat-soluble vitamins still causes precipitation of protein even within the final concentration less than 3%. Emulsifiers such as phospholipids and Tween-20 could be alternative agents to facilitate binding of the vitamins and protein. Homogenization with a tissuemizer and/or sonication is essential to obtain an emulsion for spray drying. Recovery of the spray dryer needs to be greatly improved before better recovery of both protein and vitamin can be achieved. The spray drying technique has been widely used for drying heat-sensitive foods, pharmaceuticals, and micro-encapsulation, because of the solvent's rapid evaporation from

the droplet (Re, 1998). Whey proteins were used as effective microencapsulating agents by spray drying and oxidation of anhydrous milk fat was significantly limited by micro-encapsulation in WPI (Moreau, 1996). Thus BLG could provide protection against oxidation during the drying procedure and storage. Once better recovery and less isomerization are achieved, spray drying may be used to prepare BLG-vitamin complexes more efficiently on a large scale.

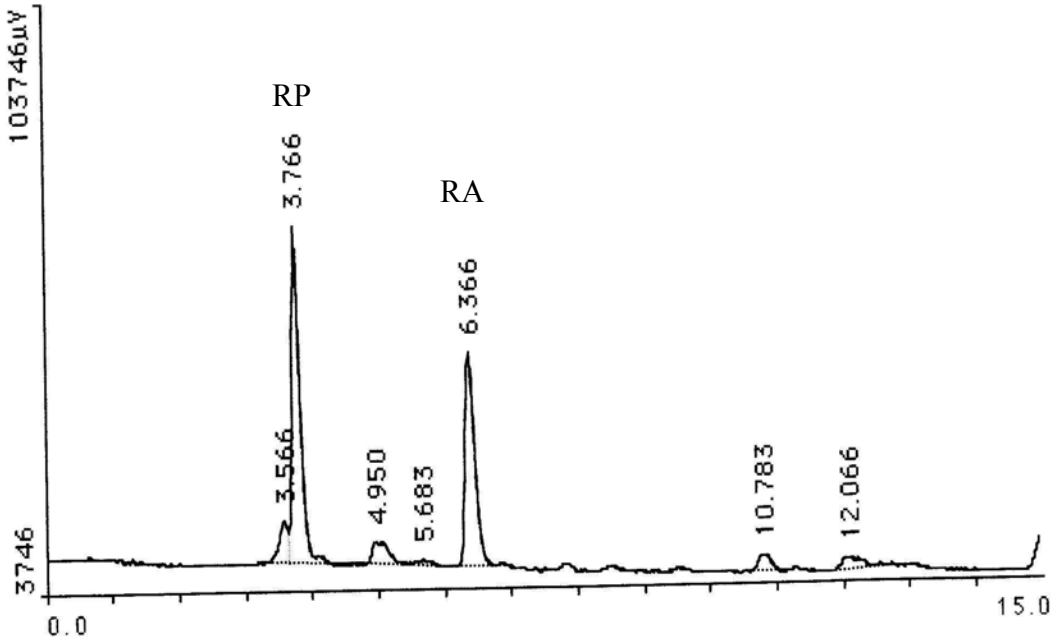

Figure 3.3 HPLC chromatogram of retinyl palmitate extracted from spray-dried BLG-vitamin A complex with retinyl acetate as internal standard

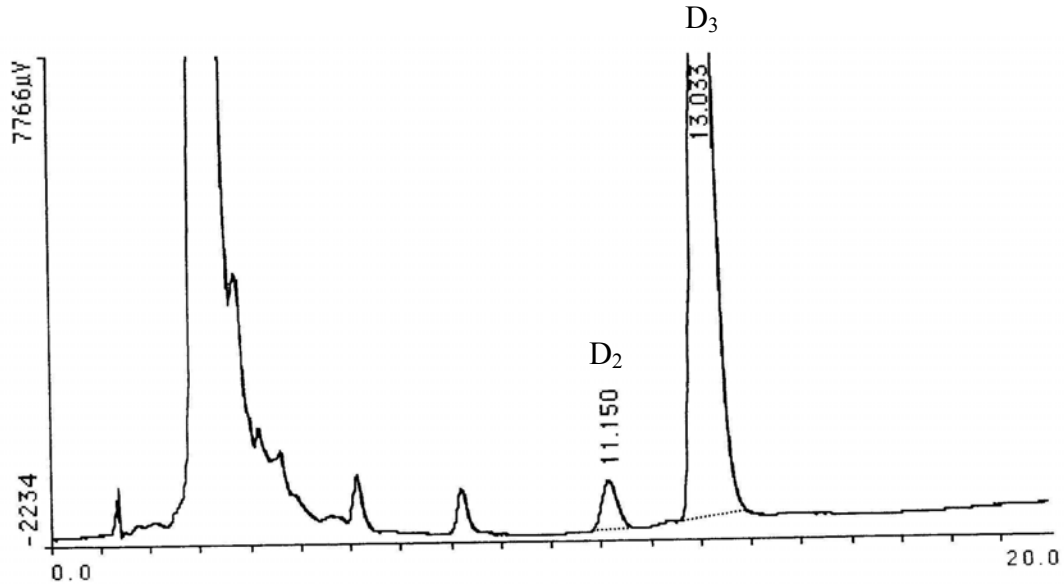

Figure 3.4 HPLC chromatogram of vitamin D_3 extracted from spray-dried BLG-D_3 complex with vitamin D_2 as internal standard

3.3 Freeze-drying

3.3.1 Introduction

In order to achieve higher recovery and less isomerization, freeze-drying was used to make dry protein-vitamin complexes. Freeze-drying is more costly and applicable to a relatively smaller scale of samples than spray drying, though it is preferred for unstable and heat labile foods or when product quality and structural integrity are critical. However, freeze-drying requires a lengthy process time and has energy costs three times those of other drying methods (Snowman, 1997).

3.3.2 Materials and Method

The freeze-drying procedure is as follows: add exact amount of Roche dry vitamins (Roche Vitamins Inc. Nutley, NJ) as retinyl palmitate and vitamin D_3 into 20 ml of a 10% BLG in water solution (protein/vitamin molar ratio 1:1); vortex to mix well. Transfer the

protein-vitamin solution into freeze-drying bottles (Labconco) and shelf freeze the solution in dry ice/ ethanol mixture in a cold bucket until completely frozen. Use Freeze Dryer 5 (Labsconco Co. Kansas City, MO). Turn the condenser on and wait until the temperature drops to 0°C. Turn on the vacuum pump, connect the bottles onto the outlets and make sure that unconnected outlets are sealed with stoppers. Switch the knob of each connected outlet from vent to vacuum. The drying procedure may take about 24 hrs for the sample to be completely dried. When it is ready, slowly turn the knob back to vent and detach the bottle from the outlet. Carefully recover the powder into a clean glass bottle covered with aluminum foil, weigh the bottle before and after filling with the powder. Assay the vitamin level in the powders, and calculate recovery rates for the protein and vitamins.

3.3.3 Results of vitamins in freeze-dried powder

Two g of beta-lactoglobulin in 20 ml DI water was mixed with 0.5 g Roche dry vitamin A (= 68.75 mg pure retinyl palmitate) and another 2 g of beta-lactoglobulin in 30 ml DI water was mixed with 0.25 g Roche dry vitamin D_3 (= 625 μg pure vitamin D_3). After freeze drying, 2.32 g vitamin A-protein complex and 2.11 g vitamin D_3-protein complex were recovered. The recovery was approximately 93%. Vitamin contents in both powders were assayed using the extraction procedures and HPLC conditions described in section 4.2. The retention times are 3.60 min and 5.67 min for retinyl palmitate and retinyl acetate, and 9.67 min and 11.17 min for vitamin D_2 and D_3, respectively (Fig.3.6 and Fig.3.7). Concentrations were interpolated from the standard curves according to the AUC ratios of RP/RA and D_3/ D_2. On average one mg vitamin A-protein powder contains 34.9 μg retinyl palmitate and one mg vitamin D_3-protein powder contains 0.339 μg of vitamin D_3 (Table 3.1).

Table 3.1 Concentrations of vitamins in the freeze-dried protein-vitamin powders

Powder weight (mg)	RP AUC	RA AUC	RP/RA ratio	μg RP/mg powder
1.2	6422167	250450	25.6	34.4
1.2	5525698	235145	23.5	31.5
1.1	6107268	230839	26.5	38.8
Powder weight (mg)	**D_2 AUC**	**D_3 AUC**	**D_3/ D_2 ratio**	**μg D_3/mg powder**
4.2	10995	115539	10.5	0.35
3.1	11149	91181	8.2	0.37
3.5	22015	160888	7.3	0.30

3.3.4 Confirmation of vitamins A and D binding with beta-lactoglobulin in the freeze-dried protein-vitamin complexes

Fluorescence spectroscopy was used to show a change in beta-lactoglobulin (BLG) structure as a result of vitamin binding. Eighteen mg each of BLG, BLG-VitA and BLG-VitD were weighed into 4.5 ml phosphate buffer (pH 7.0). The exact protein concentration in each solution was determined on the Genesys 2 spectrometer (Spectronic, Thermo Electron Scientific Instruments Co. Madison, WI) by using a molecular absorption coefficient $\in_{278}$ = 17600. The solutions were diluted to 10 μM. On the System 3 Scanning Spectrofluorometer (Optical Technology Devices, Inc.), fluorescence spectroscopy was performed using excitation wavelength 287 nm and fluorescence emission was measured at 23°C with a cell path length 1 cm, a bandwidth of 10 nm, and a scan speed of 50 nm/min. Fluorescence enhancement was detected in both BLG-VitA and BLG-VitD solutions at wavelength 287 nm and quenching was detected around 332 nm in both solutions, which indicated the binding of the vitamins and protein (Fig.3.5).

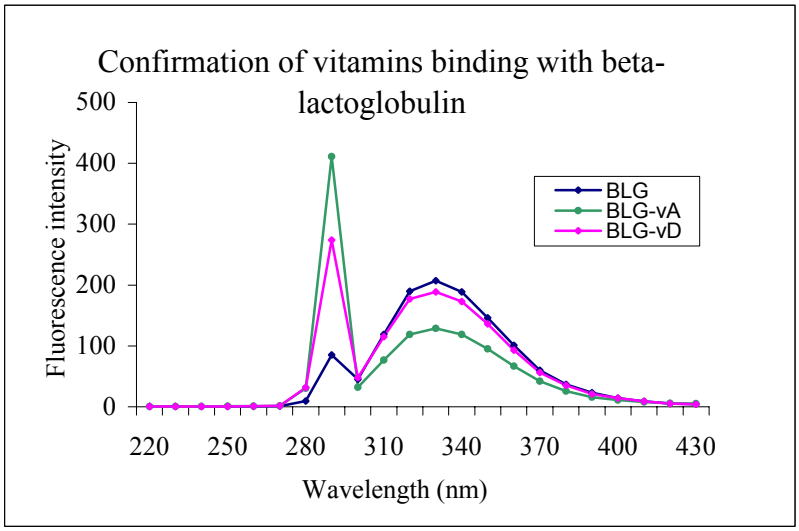

Figure 3.5 BLG-vitamins binding in freeze-dried powder

3.3.5 Discussion and conclusion

The fluorescence study verified the binding of the vitamins with BLG in the freeze-dried powder (Fig.3.5). The HPLC chromatograms (Fig.3.6 and Fig.3.7) showed no cis-isomers present in either powder. Compared with spray drying, freeze-drying yielded protein-vitamin mixes with a much higher recovery and efficiently protected heat and light sensitive vitamins from deteriorating and isomerizing. Spray-drying and freeze-drying have also been compared for beta-carotene encapsulation and preservation by Desobry et al (1997). In this study, freeze-tray drying was a more efficient method to prepare protein-vitamin complexes.

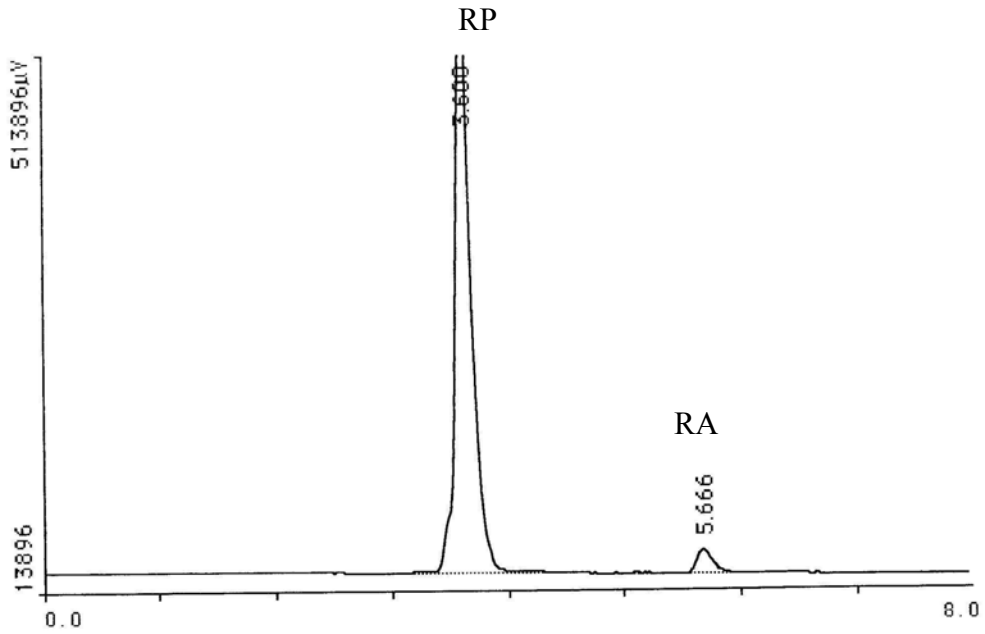

Figure 3.6 HPLC chromatogram of retinyl palmitate extracted from freeze-dried BLG-vitamin A complex with retinyl acetate as internal standard

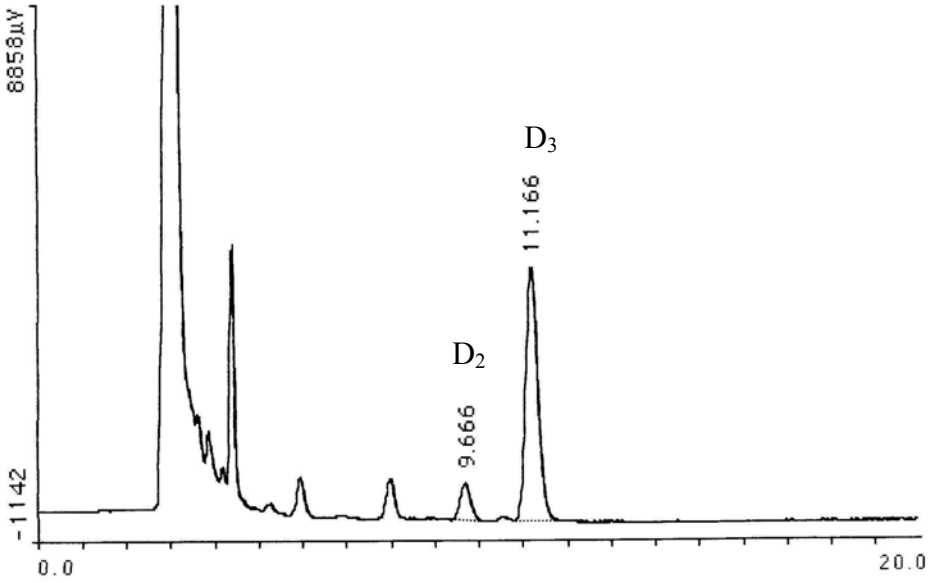

Figure 3.7 HPLC chromatogram of vitamin D_3 extracted from spray-dried BLG- D_3 complex with vitamin D_2 as internal standard

Chapter 4 HTST and UHT shelf life

4.1 Introduction

Multiple studies found that a high percentage of fortified milk products failed to meet label claims and most of the milk out of compliance was under-fortified (Holick, 1992 and Nichols, 1991). Loss of vitamins often occurred during processing and storage (Tanner, 1988). Vitamins are sensitive to light, heat and oxygen. The presence of milk fat appears to protect against vitamin degradation, therefore skim milk tends to be under-fortified when fortified with oil-based fortifiers. BLG is proposed to be a stable and protective fortifier in skim milk based on its capacity of binding vitamins A and D. The purpose of this experiment was to compare the stability of BLG-complexed vitamins with that of the oil-based vitamins. The influence of HTST or UHT pasteurization, containers, and fortifiers on shelf-life stability of vitamins was studied. Vitamin levels in milk were assayed by HPLC that is a predominant method for separation and quantification of retinoids and vitamin D in biological samples. HPLC methodology is described in detail in this chapter. Results from shelf-life studies were discussed and compared with other studies.

4.2 Vitamin extraction and HPLC analyses

4.2.1 Materials

Vitamin fortified skim milk was supplied by the North Carolina State University dairy plant. Pure vitamin standards were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals and solvents were purchased from Fisher (Pittsburgh, PA) and were of the highest analytical quality. Regular equipment included BUCHI Rotavapor R-124 (BUCHI Analytical Instruments), N-Evap N_2 dryer (Mayer Analytical Co.), Genie Vortex mixer

(Fisher Scientific, Raleigh, NC), shaker (New Brunswic Scientific), Centrific Centrifuge (Fisher Scientific), and AC210 MCI analytic balance (Sartorius Inc., Edgewood, NY).

4.2.2 Assay of retinyl palmitate in fortified skim milk

4.2.2.1 Sample preparation and storage

Samples were sub-sampled into 2 ml aliquots in screw top round bottom tubes. They were kept refrigerated until analyzed. Two replicate analyses were performed on each milk sample. One μg of retinyl acetate as internal standard was spiked into each 2-ml skim milk sample. Five ml of absolute ethanol was then added as the precipitating solvent. The tubes were vortex mixed and allowed to stand for 5 minutes. Any tubes that leak were marked. Five ml of hexanes was added as the extracting solvent and tubes were vortex-mixed 1 minute, 3 times, with 5 minutes standing between mixing. Three ml of distilled water was added and tubes were inverted 3 times. Tubes were centrifuge 10 minutes afterwards, and one aliquot of the hexane layer was transferred into amber sample vials. The samples were kept at –20 °C before HPLC analysis (Woollard, 1986).

4.2.2.2 HPLC analysis

HPLC analyses were performed on a Waters 510 pump system with UVIS 203 detector and U6K manual loading injector (Waters Associates, Milford, MA). The 4.6 x 250-mm 5 μm silica column and guard column (Prodigy, Phenomenex, Inc., Torrance, CA) were used at room temperature. The isocratic mobile phase hexane/isopropanol (99.6: 0.4, v/v) was run at a flow rate of 1 ml/min. The wavelength λ_{max} = 325 nm was used to quantitate the results. Data were integrated by using a Dynamax HPLC data processing program (Rainin Instrument Co., Woburn, MA).

4.2.2.3 Standard curve and recovery study

Table 4.1 Preparation of standard curve and recovery study for retinyl palmitate in skim milk

Sample #	Retinyl Palmitate Stock (125 μg/ml, 8 μl = 1 μg)	Retinyl Acetate Stock (172 μg /ml, 5.8 μl = 1 μg)
#1	8 μl	5.8 μl
#2	16 μl	5.8 μl
#3	24 μl	5.8 μl
#4	32 μl	5.8 μl

For the standard curve, the above stock solutions were spiked accurately into 4 separate tubes (Table 4.1). The solvents were evaporated under nitrogen until dry. Two ml of hexane was added to each tube afterwards and vortexed before 25 μl was injected for HPLC analysis. For the recovery study, the above stock solutions (Table 4.1) were accurately spiked into another 4 tubes containing 2 ml raw skim milk each. Vitamin A was extracted according to the protocol described in section 4.2.2.1. Hexane layers were filtered and ready for HPLC analysis. Raw skim milk without spike served as control. Area under the curve (AUC) from the HPLC detector was recorded for both Retinyl Palmitate (RP) and Retinyl Acetate (RA) in each sample. The standard curve for retinyl palmitate in fortified skim milk is shown in Fig. 4.1. The recovery for retinyl acetate and retinyl palmitate was included in Tables 4.2 and 4.3. Typical HPLC chromatograms are shown in Fig. 4.2. The retention times for retinyl palmitate and retinyl acetate are 3.5 min and 5.3 min, respectively.

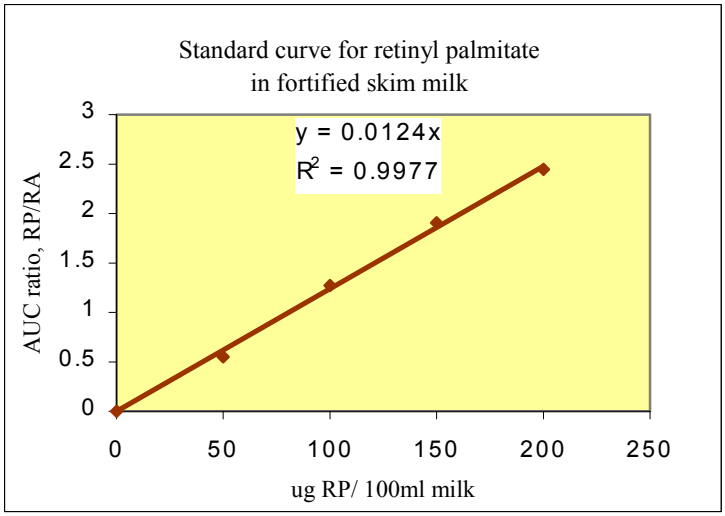

Figure 4.1 Standard curve for retinyl palmitate in fortified skim milk

Table 4.2 The recovery of retinyl acetate

Sample #	AUC in standard curve	AUC in recovery study	Recovery
#1	480402	460628	0.959
#2	496257	473790	0.955
#3	476757	451421	0.947
#4	469484	448230	0.955
Mean = 0.954; Standard Deviation = 0.005			

Table 4.3 The recovery of retinyl palmitate

Sample #	AUC in standard curve	*AUC in recovery study	Recovery
#1	264399	285900	1.081
#2	631200	578342	0.916
#3	909264	821870	0.904
#4	1148763	1097624	0.955
Mean = 0.964; Standard Deviation = 0.081 * AUC in recovery study = AUC in Sample – AUC in Control.			

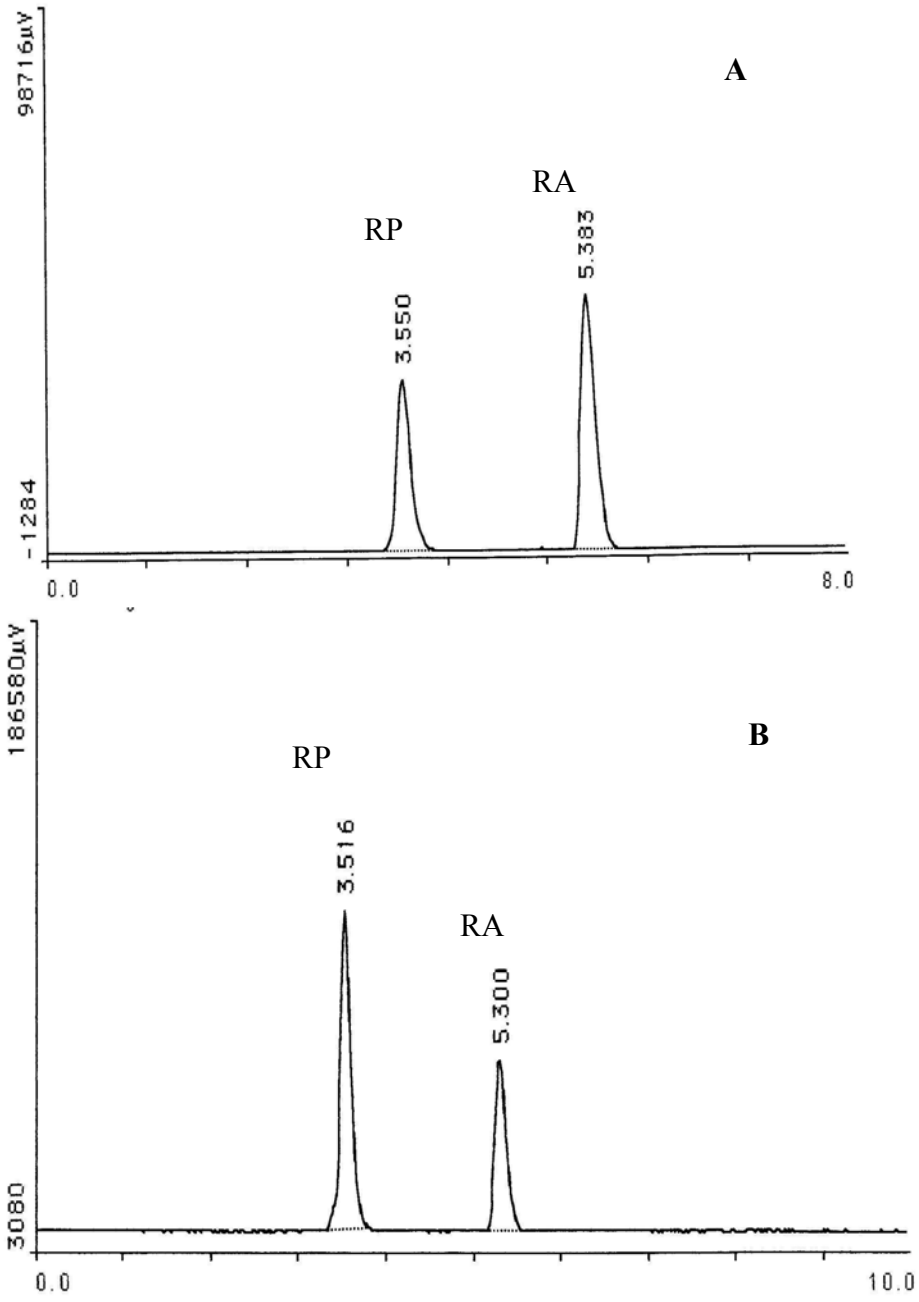

Figure 4.2 HPLC chromatograms of retinyl palmitate with retinyl acetate

A: Standards; **B**: extract of fortified skim milk with internal standard.

4.2.3 Assay of vitamin D_3 in fortified skim milk

4.2.3.1 Sample preparation and storage

Samples were sub-sampled into 15 ml aliquots in the test tubes and kept refrigerated until analyzed. Two replicate analyses were performed on each milk sample. Samples were spiked with 100 µl D_2 working solution as internal standard because the samples were fortified with D_3. Fifteen ml of 1% ethanolic pyrogallol was added as an antioxidant. Samples were cooled in an ice bath, and ~6.0 g of KOH pellets were added under a stream of N_2. Samples were kept in the ice bath until the KOH was dissolved. The tubes were shaken at room temperature in the dark for at least 18 hours.

After the above cold saponification steps, the sample was transferred to a 125 ml separatory funnel and 15 ml of distilled water, 5 ml of absolute ethanol, and 45 ml of hexanes were added. Samples were shaken for 1 minute and allowed to stand for 4 minutes. The aqueous layer was transferred to an Erlenmeyer flask and the organic layer was transferred to a 250 ml separatory funnel. The aqueous layer was returned into the 125 ml separatory funnel, 45 ml hexanes added and the mixture was shaken 1 minute, and allowed to stand 4 minutes. The last two steps were repeated once. The aqueous layer was then discarded. Fifty ml of 5% KOH was added to the combined organic layer in the 250 ml funnel. Samples were shaken 1 minute, allowed to stand 4 minutes, and the aqueous layer was discarded. Fifty ml of distilled H_2O was then added to the funnel. Samples were shaken 1 minute, allowed to stand 4 minutes, and the aqueous layer was discarded. Fifty ml of 55% ethanol was added to the 250 ml funnel. Samples were shaken 1 minute, allowed to stand 10 minutes, and the aqueous layer was discarded. The organic layer was collected into a rota-vap tube and evaporated to dryness at 50°C. Five ml of hexane was immediately added into the tube with

the dried residue of vitamin D. The tube was swirled and the rinse was transferred to a centrifuge tube. The tube was then washed twice, first with 3 ml hexane and then with 1 ml hexane. The washes were collected into the centrifuge tube. The solvent was removed under a stream of N_2 and the residue was re-dissolved in 500 µl methanol and filtered through 0.45 µm syringe filter into a 2 ml amber vial. Samples were kept at –20 °C before HPLC analysis (Murphy, 2001).

4.2.3.2 HPLC analysis

HPLC analyses were performed on a Waters 510 pump with an automated gradient controller, a UVIS Linear detector, and a U6K manual loading injector (Waters Associates, Milford, MA). A reversed-phase, 4.6 x 250-mm Vydac TP201 C18, 5 µm column with a guard column (Vydac, Hesperia, CA) was used at room temperature. The mobile phase acetonitrile/ethyl acetate/chloroform (88:8:4, v/v/v) was run at a flow rate of 1 ml/min set in the following flow program including column conditioning and equilibration: A = acetonitrile/ethyl acetate/chloroform (88:8:4, v/v/v); B = Methanol.

Time	Flow	%A	%B
0.0	1.0	100	0
22.0	1.0	100	0
23.0	2.5	0	100
29.0	3.0	0	100
31.0	2.5	100	0
35.0	3.0	100	0
35.5	1.0	100	0
36.0	1.0	100	0

4.2.3.3 Standard Curve and recovery study

Table 4.4 Preparation of standard curve and recovery study for vitamin D_3

Sample#	**D_3 working solution** (2.08 μg/ml, 24 μl = 0.05 μg)	**D_2 working solution** (1.74 μg /ml, 90 μl = 0.16μg)
#1	24 μl	90 μl
#2	48 μl	90 μl
#3	96 μl	90 μl
#4	192 μl	90 μl

For the standard curve, the D_2 and D_3 solutions were spiked accurately into 4 separate tubes according to table 4.4. Methanol was added to 0.5 ml volume for each tube. Fifty μl of each sample was injected for HPLC analysis. For the recovery study, the D_2 and D_3 solutions were spiked into 3 tubes with 15 ml milk each, labeled as Sample #2- #4. Vitamin D was extracted following the protocol as described in section 4.2.3.1. Hexane layers were dried, dissolved in 0.5 ml methanol and filtered. Fifty μl of each sample was injected for HPLC analysis. Raw skim milk without spike served as control. Area under the curve (AUC) was recorded for both vitamins D_2 and D_3 in each sample. The standard curve is shown in Fig. 4.3. The Recovery of vitamins D_2 and D_3 was included in tables 4.5 and 4.6. Typical HPLC chromatograms are shown in Fig. 4.4. The retention times for vitamin D_2 and vitamin D_3 are 11.6 min and 13.7 min, respectively.

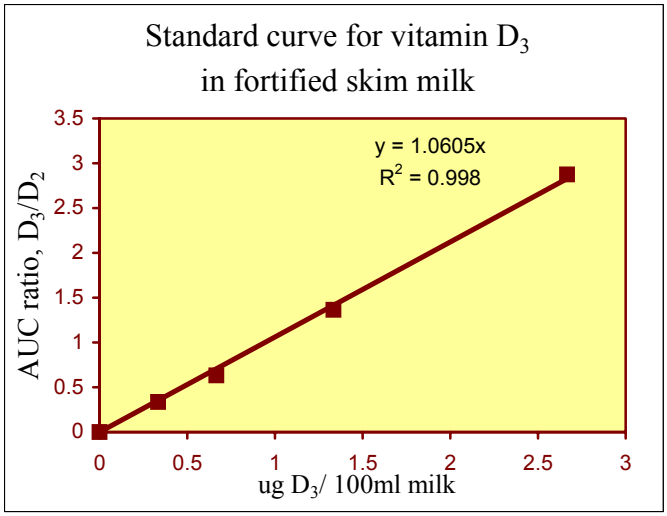

Figure 4.3 Standard curve for vitamin D_3 in fortified skim milk

Table 4.5 The recovery of vitamin D_3

Sample #	AUC In standard curve	*AUC in recovery study	Recovery
#2	23686	21973	0.928
#3	22677	21742	0.959
#4	23987	21098	0.880
Mean = 0.922; Standard Deviation = 0.040 * AUC in control = 0			

Table 4.6 The recovery of vitamin D_2

Sample #	AUC In standard curve	*AUC in recovery study	Recovery
#2	14974	14551	0.972
#3	30924	29476	0.953
#4	68934	59158	0.858
Mean = 0.928; Standard Deviation = 0.060 * AUC in control = 0			

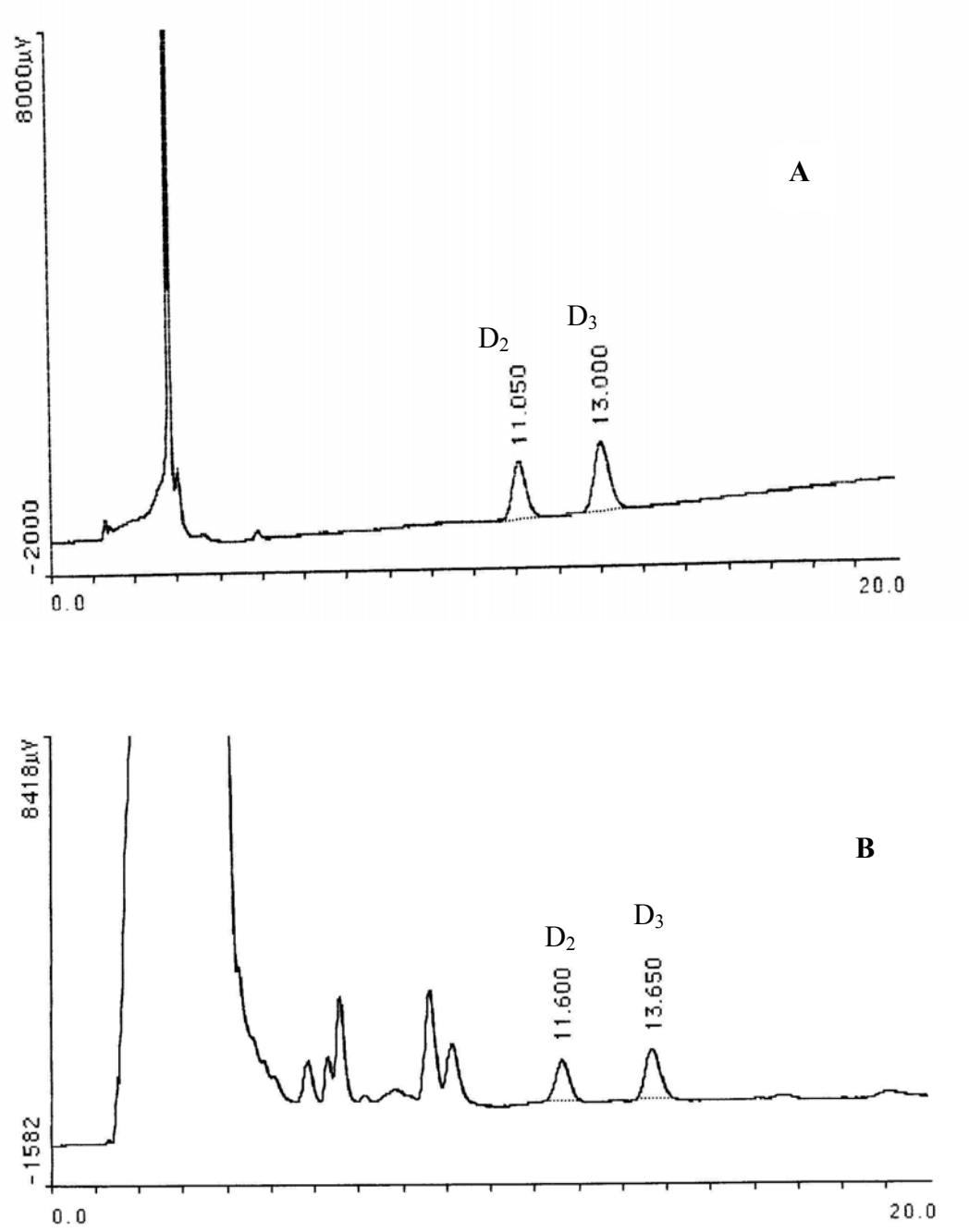

Figure 4.4 HPLC chromatograms of vitamin D_3 and vitamin D_2

A: Standards; **B**: Extract of fortified skim milk with added internal standard

4.2.4 Discussion

The extraction methods and HPLC programs described in sections 4.2.2.1 and 4.2.3.1 had produced excellent resolution and recovery for vitamins A and D from fortified skim milk with the internal standards (Figures 4.2 and 4.4), which was consistent with other studies using similar methods (Faulkner, 2000 and Murphy, 2001). Internal standards showed equal recovery to the analytes. Further, vitamins A and D were well separated from interfering substances in milk samples. There are no interfering peaks on the vitamin A sample HPLC chromatograms when compared to the standards. There were multiple polar compounds eluted before vitamins D_2 and D_3 on the vitamin D sample HPLC chromatograms, which could be interfering lipids from the milk samples, but they did not affect the resolution of the analytes. The absence of solid-phase clean up step during vitamin D extraction seemed to have no effect on the analysis of vitamin D in skim milk. However, saponification turned out to be the indispensable step to achieve good recovery and resolution (data not shown). Though an auto-sampler will greatly reduce the sampling variation, in general, these methods were easy and reproducible for the analyses of vitamins A and D in fluid milk on a regular basis.

4.3 HTST skim milk shelf life

4.3.1 Fortification and HTST pasteurization of skim milk

Spray-dried vitamin-protein mixes were added into raw skim milk from the NCSU dairy plant to achieve a fortification level of 116.3 μg (211 IU) retinyl palmitate /100 ml milk and 1.06 μg (42.3 IU) vitamin D_3 /100 ml milk. The fortified milk was mixed well with stirring bars. Milk was sub-sampled into 20-ml aliquots in sterilized screw top glass test tubes and

Nalgene polyethylene tubes. HTST pasteurization was achieved in a heating system with two water baths. Milk in the tubes was immersed in a 99°C constant temperature water bath (Mode-M1, Cannon Instrument Co.) for 1 min, resulting in rapid heating of milk to 72-77°C; the tube was then immediately moved to a water bath of 75°C for 15 sec. The processed milk in tubes was then rapidly cooled in an ice bath and kept in a refrigerator for the vitamin shelf life study (Chen, 1998). Roche dry vitamin mixes were also used to fortify raw skim milk as a reference. Oil-based-vitamin fortified skim milk that was obtained from the NCSU dairy plant prior to pasteurization was HTST pasteurized in the lab and sub-sampled in the same procedure as above. Oil-based-vitamin fortified skim milk pasteurized in the dairy plant was sub-sampled directly. The concentration of retinyl palmitate in oil-based vitamin-fortified skim milk stored in sterilized paperboard carton was measured during a 32-day shelf life study.

4.3.2 Results

Assay for vitamin levels in HTST fortified skim milk was performed during a 4-week or 32-day shelf life study. One aliquot of milk was taken out of the refrigerator right before the assay. Extraction procedure and HPLC methodology were the same as that addressed in sections 4.2.2.1 and 4.2.3.1.

During this 32-day shelf life study retinyl palmitate level was tested in skim milk fortified with Roche dry vitamin A and spray dried BLG-vitamin-A complex in glass and plastic bottles. The abbreviations used in this chapter are as follows: **R-G** = Roche in glass; **R-P** = Roche in plastic; **S-G** = Spray-dried powder in glass; **S-P** = Spray-dried powder in plastic. Area Under the Curve (AUC) was recorded for retinyl palmitate (RP) and retinyl

acetate (RA), and RP/RA AUC ratio was obtained. Concentration of retinyl palmitate was interpolated from the standard curve in section 4.2.2.2.1. Data are shown in Table 4.7 and Figure 4.5. Retinyl palmitate dropped from 175 μg / 100 ml in R-G samples and 159.27 μg / 100 ml in R-P samples to 31.45 μg / 100 ml (more than 80% loss), while it was stable in S-P and S-G samples during the 32-day shelf life. But the original level in spray-dried powder fortified milk was only 1/3 of the targeted fortification.

Table 4.7 Retinyl palmitate in Roche dry vitamin and spray-dried BLG-vitamin complex - fortified skim milk during the 32-day shelf life

Units / Time	**Day 1**	**Day 2**	**Day 4**	**Day 8**	**Day 16**	**Day 32**
R-G (μg/100ml)	[1]175.00	175.00	164.52	166.94	102.42	31.45
	[2] *100%*	*100%*	*94%*	*95%*	*58.5%*	*18%*
R-P (μg/100ml)	159.27	166.13	169.35	165.32	46.77	31.45
	100%	*104%*	*106%*	*104%*	*29.4%*	*18%*
S-G (μg/100ml)	55.65	56.05	53.23	55.65	53.63	54.44
	100%	*101%*	*95.7%*	*100%*	*96.4%*	*97.8%*
S-P (μg/100ml)	55.65	56.45	58.87	52.42	51.21	52.02
	100%	*101%*	*106%*	*94.2%*	*92%*	*93.5%*

[1] Each value is the mean of two duplicate measurements.
[2] *Italic* numbers signify percentage of vitamin remaining relative to the first day of shelf life.

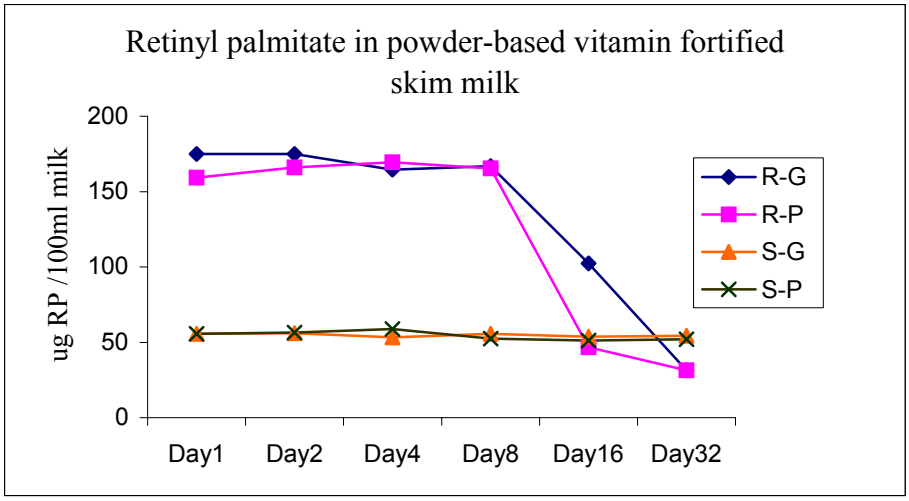

Figure 4.5 Time series of RP in powder-based vitamin fortified skim milk

Table 4.8 Vitamin D_3 in Roche dry vitamin and spray-dried BLG-vitamin complex - fortified skim milk during the 32-day shelf life

Units / time	Day 1	Day 6	Day 12	Day 24	Day 32
R-G (µg/100ml)	[1] 2.36	2.07	1.98	1.89	2.20
	[2] *100%*	*87.7%*	*83.9%*	*80%*	*93.2%*
R-P (µg/100ml)	2.73	2.49	2.64	2.69	2.59
	100%	*91.2%*	*96.7%*	*98.5%*	*94.9%*
S-G (µg/100ml)	2.55	2.59	2.36	2.69	2.36
	100%	*102%*	*92.5%*	*105%*	*92.5%*
S-P (µg/100ml)	2.59	2.26	2.04	2.17	2.26
	100%	*87.3%*	*78.8%*	*83.8%*	*87.3%*

[1] Each value is the mean of two duplicate measurements.
[2] *Italic* numbers signify percentage of vitamin remaining relative to the first day of shelf life.

Also during the 32-day shelf life, vitamin D_3 level was tested in skim milk fortified with Roche dry vitamin D_3 and spray dried BLG-vitamin-D complex in glass and plastic bottles. Area Under the Curve (AUC) was recorded for vitamin D_3 and for the internal standard vitamin D_2, and D_3/D_2 AUC ratio was obtained. Concentration of vitamin D_3 was calculated from the standard curve based on the D_3/ D_2 AUC ratio. Data are shown in Table 4.8 and

Figure 4.6. Vitamin D concentration remained relatively constant during the study in all samples.

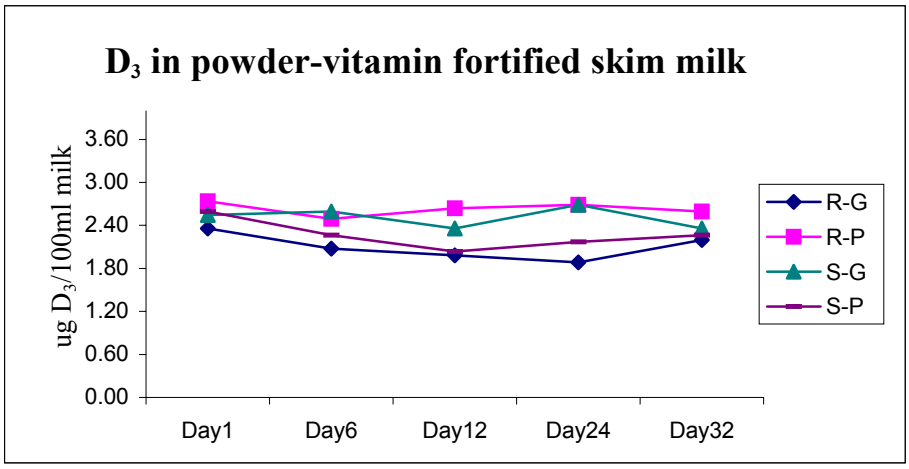

Figure 4.6 Time series of vitamin D_3 in powder vitamin fortified skim milk

Vitamin D_3 level was also measured in oil-based vitamin-fortified skim milk during a 28-day shelf life. Milk was pasteurized in the NCSU dairy plant or in the lab and stored in glass or plastic bottles. No significant decrease in vitamin D_3 concentration was detected in any samples during the shelf life study. Higher vitamin D_3 level was found in the Lab HTST-glass samples (Table 4.9 and Figure 4.7).

Retinyl palmitate level was measured in oil-based vitamin-fortified skim milk during a 28-day or 32-day shelf life. Milk was pasteurized in the NCSU dairy plant or in the lab and stored in glass, plastic bottles, or paperboard cartons. No appreciable decrease in vitamin A concentration was detected in most samples during the shelf life except for Lab HTST-glass samples, in which vitamin level dropped from an initial value of 79.84 μg / 100 ml to 17.74 μg / 100 ml on Day 32 (Table 4.10, Figure 4.8 and 4.9).

Table 4.9 Vitamin D_3 concentration in oil-base-vitamin-fortified skim milk during the shelf-life study [1]

Units / Time	Day 1	Day 7	Day 14	Day 21	Day 28
Lab HTST-glass (µg/100ml)	1.25	1.31	1.31	1.39	1.37
	[2] *100%*	*105%*	*105%*	*111%*	*110%*
Lab HTST-plastic (µg/100ml)	1.30	1.23	1.12	1.10	1.13
	100%	*94.6%*	*86.2%*	*84.6%*	*86.9%*
Plant HTST-glass (µg/100ml)	1.19	1.17	1.16	1.10	1.07
	100%	*98.3%*	*97.5%*	*92.4%*	*89.9%*
Plant HTST-plastic (µg/100ml)	1.19	1.21	1.21	1.12	1.20
	100%	*102%*	*102%*	*94.1%*	*101%*

[1] Each value is the mean of two duplicate measurements.
[2] *Italic* numbers signify percentage of vitamin remaining relative to the first day of shelf life.

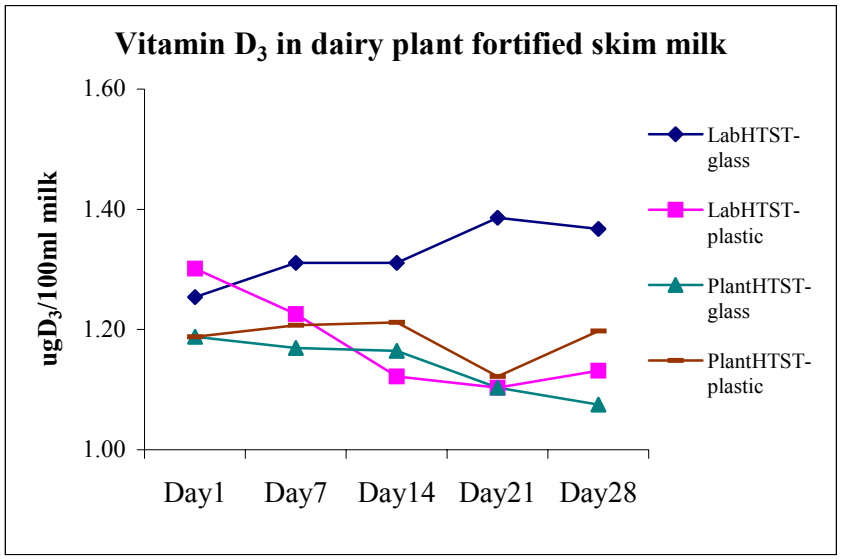

Figure 4.7 Time series of vitamin D_3 in dairy plant fortified skim milk

Table 4.10 Retinyl palmitate concentration in oil-based vitamin fortified skim milk during the 32-day shelf life study

Units / Time	Day 1	Day 7	Day 14	Day 21	Day 28	
PlantHTST-glass (µg/100ml)	[1]145.16	143.55	142.74	138.71	138.71	
	100%	*98.9%*	*98.3%*	*95.5%*	*95.5%*	
PlantHTST-plastic (µg/100ml)	157.26	152.42	137.90	136.29	138.71	
	100%	*96.9%*	*87.7%*	*87.1%*	*88.2%*	
Units / Time	**Day 1**	**Day 2**	**Day 4**	**Day 8**	**Day 16**	**Day 32**
LabHTST-glass (µg/100ml)	79.84	79.03	75.00	58.06	58.06	17.74
	100%	*99.0%*	*93.9%*	*72.7%*	*72.7%*	*22.2%*
LabHTST-plastic (µg/100ml)	75.00	61.29	66.94	64.52	62.10	57.26
	100%	*81.7%*	*89.3%*	*86%*	*82.8%*	*76.3%*
Paperboard carton (µg/100ml)	126.61	129.03	125.81	124.19	112.10	112.10
	100%	*102%*	*99.4%*	*98.1%*	*88.5%*	*88.5%*

[1] Each value is the mean of two duplicate measurements.
[2] *Italic* numbers signify percentage of vitamin remaining relative to the first day of shelf life.

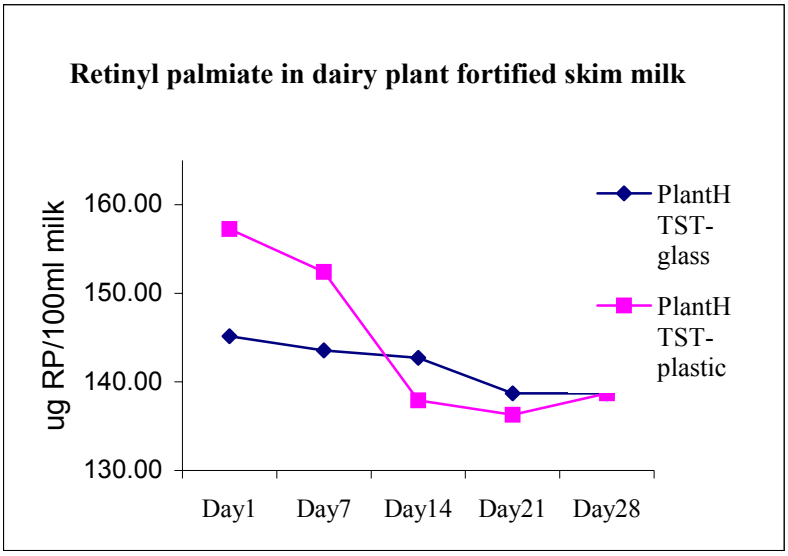

Figure 4.8 Retinyl palmitate concentration in dairy plant fortified skim milk during the 28-day shelf life

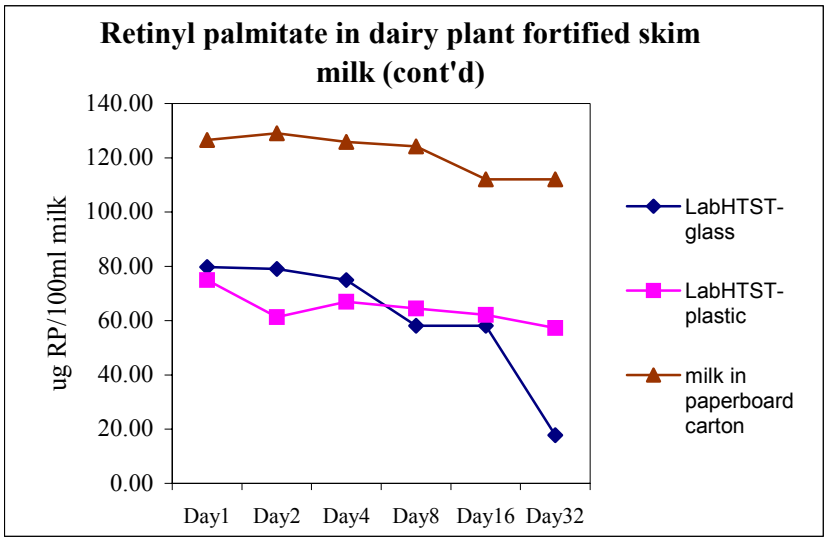

Figure 4.9 Retinyl palmitate concentration in dairy plant fortified skim milk during the 32-day shelf life

4.3.3 Discussion and Conclusion

This experiment did not observe appreciable changes of vitamin D_3 concentration in milk with various types of fortification, indicating that vitamin D_3 is more stable than vitamin A palmitate in fortified skim milk, as shown in other studies (Indyk, 1996). Retinyl palmitate level did not change significantly in oil-based vitamin-fortified skim milk after homogenization and pasteurization in the NCSU dairy plant, and storage in either glass bottles or plastic/ paperboard cartons. However, 16 days after HTST pasteurization with the lab-scale method, a significant decrease in retinyl palmitate occurred in oil-based vitamin-fortified skim milk. These samples were taken from the dairy plant before homogenization and pasteurization but after fat separation and stored in glass test tubes, suggesting that efficient homogenization played an important role in preventing vitamin loss from degradation, separation, or adherence to the container. The reason that retinyl palmitate level remained unchanged in Nalgene plastic tubes could be inactivation of free radicals by plastic

materials. Smith et al (1988) reported 35% reduction in vitamin A in total parenteral nutrition admixture without intra-lipid after 48 hr at 5°C and 60% reduction after 48 hr at 25°C due to absorption of the vitamin to the polyethylene material. In this experiment, package materials did not affect shelf life stability of vitamin A when the homogenized samples were stored in a refrigerator, which disagreed with the theory that vitamins A and D in skim milk would be absorbed onto polyethylene or paperboard bottles (Paredes, 1996). Retinyl palmitate concentration decreased significantly in sample fortified with Roche dry vitamin A palmitate at Day 16 in both glass and plastic bottles. The vitamin level was even lower in plastic than glass bottles, indicating precipitation, sorption or deterioration of the vitamin. Vitamin A palmitate in sample fortified with the spray-dried BLG-vitamin complex kept a relatively stable concentration level during the shelf life study. The lower initial concentration in these samples was probably due to oxidation of vitamin on the protein surface after the spray-drying process. In summary, vitamin D_3 is quite stable in fortified HTST processed skim milk; Roche dry vitamin A powder is not stable after 2 weeks of shelf storage; and spray-dried BLG- retinyl palmitate complex exhibits good stability in HTST pasteurized skim milk made with a lab-scale procedure.

For future work, noting that light exposure during shelf life can detrimentally affect vitamin stability and flavor quality of milk in HDPE containers (Whited, 2002), comparison of regular oil-based and BLG-based vitamins can be made in whole, low-fat and skim milks under conditions simulating that in supermarkets, which would test the ability of BLG to protect vitamin deterioration under light exposure.

4.4 UHT Skim milk shelf life

4.4.1 UHT processing of skim milk

Skim milk from the NCSU dairy plant contains approximately 0.2% milk fat after leaving the separator. Right before homogenization (APV Gaulin 300CGD, Tonawanda, NY), the fortifiers were mixed thoroughly into raw skim milk. Given that one-kilogram of AIN-93G pelleted rodent diet contains 4000 IU retinyl palmitate and 1000 IU vitamin D, the fortification levels for vitamins A and D in skim milk were calculated as 147 IU / 100 ml and 36.7 IU / 100 ml, respectively, based on the milk consumption data from the animal pilot study (section 5.2.2). To make 150% fortification level (to account for anticipated losses in UHT processing), 30 mg pure retinyl palmitate and 344 μg pure vitamin D_3 (in the form of freeze-dried BLG-vitamin complexes) were added to 25 L of skim milk. Thirty gallons of unfortified skim milk, 15 gallons of protein-vitamin fortified skim milk and 15 gallons of regular oil-based vitamin-fortified skim milk were UHT processed. After UHT processing at the temperature 139.4°C (283°F) for 4 sec, milk was packaged into sterilized 250 ml paperboard cartons (no-bac unitherm VIII, Waukesha Cherry-burrell, Delaven, WI). Milk was stored at room temperature for UHT shelf life and bioavailability studies.

4.4.2 Assay for vitamins in UHT skim milk by HPLC

Assay for vitamin levels in UHT vitamin fortified skim milk was performed during a 6-week shelf life at day 1, week 1, week 3, week 4 and week 6. Data were summarized in tables and time series plots. In addition to the assay described in Chapter 4, vitamin A level was also confirmed by using the saponification procedure (Ross, 1986). Retinol instead of retinyl palmitate was measured by the following procedure: draw 2 ml (in duplicate) of milk

from unfortified skim milk (**UFM**), oil-based vitamin-fortified skim milk (**OFM**), and protein-complexed vitamin-fortified skim milk (**PFM**) into screw top test tubes. Make 100 ml saponification reagent by adding 5 g KOH, 1 g pyrogallol, and 1 ml H_2O to a flask and making up to 100 ml with ethanol. Add 4 ml of the saponification reagent into each tube; incubate in a 55°C water bath for 30 min and cool for 5-10 min. Add 4 ml hexane into the tube, and vortex 1 min. Add 2 ml of deionized water, and vortex 1 min. Centrifuge10 min to separate the layers. Retinyl acetate was then added as internal standard into the hexane layer and the samples were ready for HPLC analyses. Typical chromatograms are shown in Fig. 4.11 and 4.12. The retention times are approximately 2.78 min and 7.6 min for retinyl acetate and retinol, respectively.

4.4.3 Results for vitamins in UHT skim milk during shelf life

After UHT processing, the level of vitamin A palmitate in protein-vitamin complex-fortified skim milk dropped to 1/3 of the targeted fortification level, continued to drop until week 2, and then remained unchanged until week 6. Retinyl palmitate in regular oil-based vitamin-fortified skim milk started to drop at week 2 and reached 65% of the original level at week 6. Vitamin D_3 level was relatively constant in the protein-based vitamin fortified milk, and slowly decreased in oil-based vitamin-fortified milk so that the concentration at week 6 was about 50% of the original level (Table 4.11 and Figure 4.10).

Table 4.11 Vitamin A palmitate and vitamin D_3 shelf life in UHT skim milk

RP Unit / Time	Day 1	Day 3	Week 2	Week 3	Week 4	Week 6
PFM (µg/100ml)	[1] 34.68	29.03	17.74	16.94	15.32	14.52
	[2] *100%*	*83.7%*	*51.2%*	*48.8%*	*44.2%*	*41.9%*
OFM (µg/100ml)	103.23	104.84	86.29	91.94	61.29	66.94
	100%	*102%*	*83.6%*	*89.1%*	*59.4%*	*64.8%*
Vit D_3 Unit / Time	**Day 1**	**Week 2**	**Week 3**	**Week 4**	**Week 6**	
PFM (µg/100ml)	1.57	1.46	1.43	1.24	1.26	
	100%	*93.0%*	*91.1%*	*79.0%*	*80.0%*	
OFM (µg/100ml)	1.23	0.97	0.77	0.75	0.62	
	100%	*78.9%*	*62.6%*	*61.0%*	*50.4%*	

[1] Each value is the mean of two duplicate measurements.
[2] *Italic* numbers signify percentage vitamin remaining relative to the first day of shelf life.

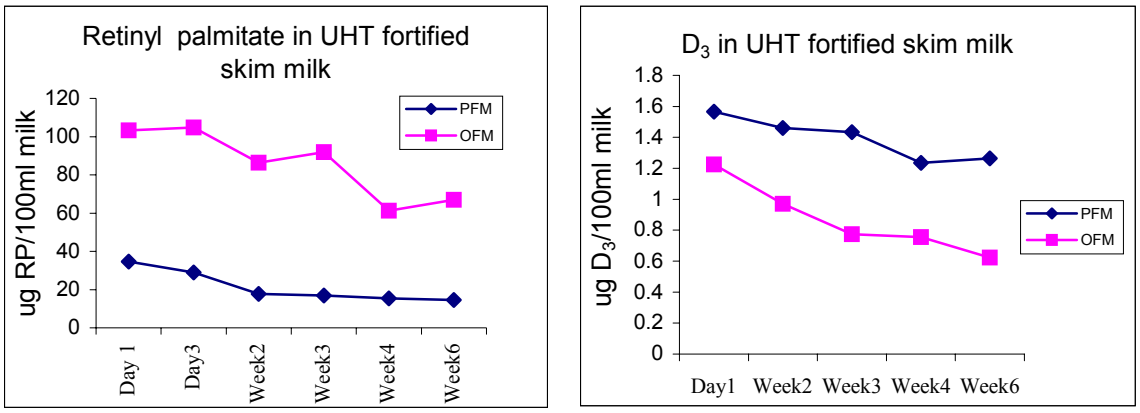

Figure 4.10 Time series of vitamin A palmitate and vitamin D_3 shelf life in UHT skim milk

4.4.4 Discussion and Conclusion

High temperature (283°F) may have partially denatured the protein during the pasteurization, which could result in vitamin A deposition at the bottom of the tank and a significant drop of vitamin A content in the final product. Possibly due to a different binding site and characteristics of vitamin D_3 binding with the protein (Wang, 1997), UHT treatment did not affect the distribution of vitamin D_3. The slow decrease of both vitamins in UHT processed skim milk might be caused by oxidative degradation by residual hydrogen peroxide from the UHT processing and sorption of vitamins onto the polyethylene material inside the paperboard cartons. Stability of vitamin A in UHT milks was studied by Lemaguer and his colleagues in 1983. Panfili and his colleagues (1998) studied the influence of thermal stress on retinol isomerization in milk. Pasteurized milks treated for 15s at temperatures ranging from 72-76 °C had an average 13-cis: all-trans retinol ratio of 6.4%, while UHT milk had a much higher degree of isomerization with a ratio of 15.7%. In contrast, they found no isomerization in raw milks. In conclusion, protein-based vitamin A palmitate is not an appropriate fortifier for UHT skim milk while β-lactoglobulin complexed vitamin D_3 is a more stable and effective fortifier than the regular oil-based fortifier.

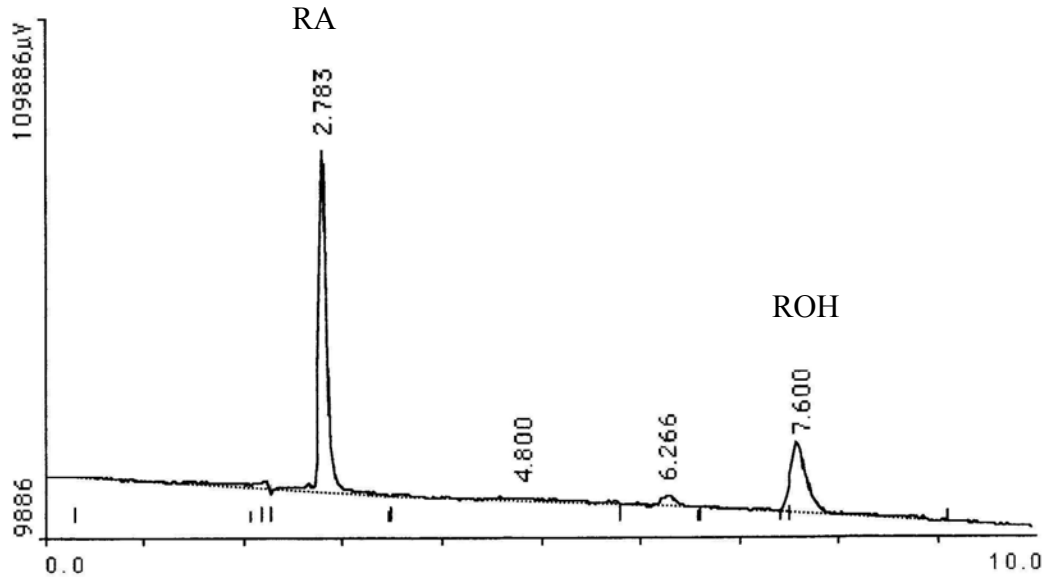

Figure 4.11 HPLC chromatogram of retinol from saponified UHT protein-based vitamin-fortified skim milk with retinyl acetate

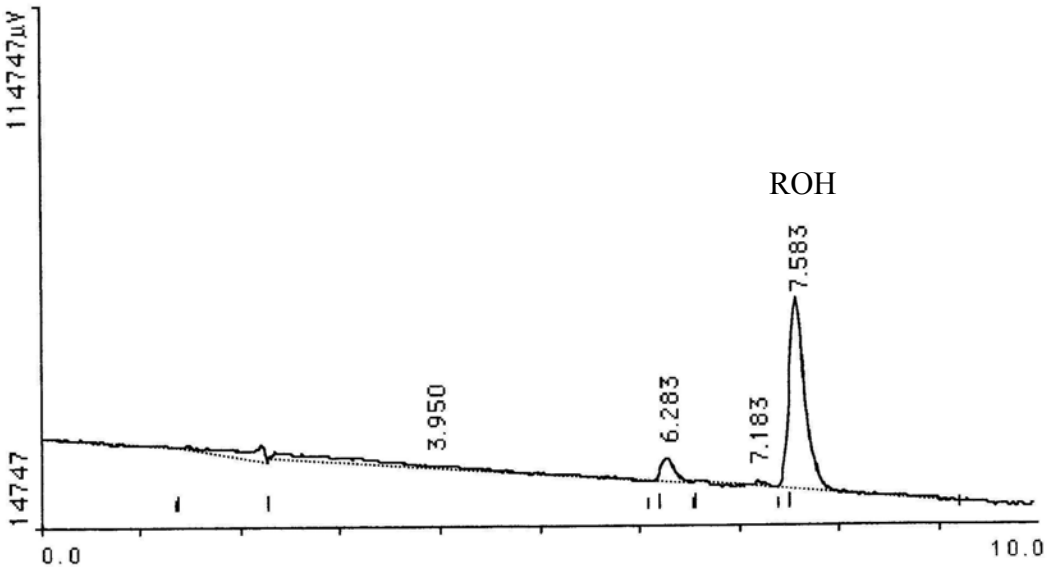

Figure 4.12 HPLC chromatogram of retinol from saponified UHT oil-based vitamin-fortified skim milk

Chapter 5 Vitamins A and D bioavailability in rats

5.1 Introduction

Despite intensive research, no definite biological function has been ascribed to BLG. From the structural and sequence similarities of BLG to retinyl binding protein (RBP), it was thought to be involved in retinol transport from mother to neonate (Papiz, 1986). BLG is resistant to the proteolytic activity in the stomach and there are intestinal receptors found specific for the protein, which suggests BLG may play a role in the delivery of retinol to the intestinal absorptive cells after hydrolysis of esterified retinol in the rumen. The effect of bovine milk BLG on intestinal uptake of retinol was examined in suckling rats with the everted gut-sac technique (Said et al, 1989). Uptake of retinol bound to β-lactoglobulin was significantly higher than that of free retinol both in the jejunum and the ileum.

The purpose of this study was to compare the bioavailability of BLG-complexed with that of oil-based vitamins A and D in rats. Milk and food consumption of rats were measured in a pilot study. Serum vitamin levels were used as indicators of the vitamin status. Serum and bone calcium, bone ash percentage and a three-point bending test were performed to indicate the effect of vitamin D status on calcium metabolism.

5.2 Experiment I - Milk consumption pilot study

5.2.1 Experimental design and methods

The purpose of this experiment was to estimate the average food and milk consumption of younger and older rats so that in experiment II milk will be provided with appropriate vitamins A and D fortification concentration to meet the requirement of the animal. Note that newborn rats consume rat milk, as their only water source from birth until weaning and the

total solids content of rat milk is much higher than cow's milk. Urine specific gravity was measured with a refractometer (Model VET360, Leica, Buffalo, NY) in both groups to detect any difference in fluid balance between milk and water groups. In a one-week feeding study, 10 5-week old rats and 10 13-week old rats (Charles River Laboratory, Raleigh, NC) were divided into two groups (water and milk) for each age. Both groups were fed a pelleted rat chow (LabDiet. Creedmoor, NC). The water groups were fed water and the milk groups were fed vitamins A and D-fortified skim milk (HTST pasteurized in the NCSU dairy plant). Body weight, food and fluid consumption were measured every day (Animal-weighing balance, Voyager V1D110, Ohaus Co. Pinbrook, NJ). At the end of the trial, blood, liver and bone samples were obtained for future analysis. For statistical analysis, one-way ANOVA were performed using SAS 8.0 software. When an overall F ratio was significant, group means were considered significantly different at $p<0.05$ as determined by the Fisher least significant difference method (LSD).

5.2.2 Results

In both 5-week and 13-week groups, rats fed milk consumed significantly more liquid and less pelleted diet than those in water groups (Table.5.1). On average, 5-week old rats consumed 51.2 g milk and 16.5 g diet per rat per day; and 13-week old rats consumed 66.0 g milk and 21.9 g diet per rat per day. The 13-week old rats gained significantly less weight than 5-week groups. There was no significant difference in urine specific gravity among any of the groups as measured by the refractometer (data not shown).

Table 5.1 Food and liquid consumption and weight gain of rats in experiment I

Parameters/groups	5-week/water	5-week/milk	13-week/water	13-week/milk
Food consumption (g/rat/day)	[1] 22.72 ± 0.72 [b]	16.50 ± 0.91 [c]	26.00 ± 1.79 [a]	21.86 ± 0.95 [b]
Liquid consumption (g/rat/day)	36.70 ± 2.10 [c]	51.18 ± 4.62 [b]	48.28 ± 5.25 [b]	66.00 ± 13.64 [a]
4-day weight gain (g)	39.18 ± 2.96 [a]	36.90 ± 7.33 [a]	27.04 ± 8.94 [b]	33.5 ± 3.66 [a, b]

[1]Data were shown as mean ± SD; Means in a row without common superscripts letters are significantly different ($p<0.05$).

5.2.3 Discussion

There was no significant difference in urine specific gravity between groups, indicating that skim milk as the only drinking source did not affect fluid balance of the animals, though liquid consumption was higher in the milk group rats. Food consumption of rats in the milk groups was significantly lower than that in water groups. The contribution of energy and nutrients from skim milk may cause rats to consume less pelleted diet. As expected, younger rats exhibited greater weight gain than older rats due to faster growth, but there was no significant difference between milk groups and water groups. Data on food and milk consumption were used to estimate food and milk needed for the bioavailability study. The volume and fortification levels of UHT processed skim milk were described in section 4.4.1

5.3 Experiment II - Bioavailability Study

5.3.1 Experimental design and methods

Weanling male Wistar rats were ordered from Charles River Laboratory (Raleigh, NC). All rats were housed in individual cages at 27°C under incandescent lighting (12 hr light/dark cycle). All rats were fed vitamins A and D-deficient AIN-93G diet (Dyets, Inc., Bethlehem,

PA) upon arrival. Five rats were sacrificed within 3 days for the baseline data. All samples were stored at -20°C until analyses. Remaining rats were fed purified vitamin A and D deficient diet 2 weeks and 5 rats were sacrificed for the serum and tissue analyses. The depletion continued for another 2 weeks, and another 5 rats were sacrificed for serum and tissue analyses. The rest of the rats were then randomly divided into the following five treatment groups:

Time/Group	P/N	N/N	N/P	N/O	N/N/O
2-week repletion	N=4	N=4	N=5	N=5	N=5
4-week repletion	N=4	N=4	N=5	N=5	N=5

P/N = +Diet/NFM, **N/N** = -Diet/NFM, **N/P** = -Diet/PFM, **N/O** = -Diet/OFM, and **N/N/O** = -Diet /NFM:OFM (2:1, v/v). **+Diet** = regular AIN-93G pelleted rodent diet; **-Diet** = AIN-93G pelleted rodent diet without vitamin A or vitamin D; **NFM** = non-fortified skim milk; **PFM** = protein-based vitamin fortified skim milk; **OFM** = oil-based vitamin fortified skim milk.

Rats in all treatments had a fresh carton of UHT-processed skim milk in a glass water bottle daily as their only water source. The milk consumed during the previous day was measured. Food consumption and rat weight gain was measured weekly. At 2 and 4 weeks after beginning the treatments, five rats from each group were sacrificed and serum and tissue samples were obtained. The rats were anaesthetized with ketamine (40-80 mg/kg BW) + xylazene (13-20 mg/kg), IP. Blood was removed by cardiac puncture using 10-ml syringes and 20 GA needles. Livers were then dissected from the exsanguinated rats and weighed. The right femur was removed for measuring bone mineral content and ash percentage and the left one was removed for bone strength measurement. Death prior to recovery from anesthesia was assured by cutting the diaphragm to open the thoracic cavity (Wortsman,

2000). We used the rat as an animal model in our studies for the following reasons: 1. Rats and humans share important similarities in that the intestine of both species contains cellular retinol-binding protein, type II (CRBP II) (Ong, 1984; 1986), which appears to play a significant role in intestinal vitamin A handling. 2. Neither rat nor human milk contains β-lactoglobulin. 3. The greatest amount of literature and previous research on vitamin A metabolism comes from rat studies. 4. Rat metabolism of vitamin D is well characterized and similar to that in humans. 5. Rats are a convenient size for tissue analysis in invasive and terminal protocols. For statistical analysis, one-way ANOVA was performed using SAS 8.0 software. When an overall F ratio was significant, group means were considered significantly different at $p<0.05$ as determined by the Fisher least significant difference method (LSD).

5.3.2 Results of body weight and nutrient consumption

5.3.2.1 Vitamin levels in regular and deficient AIN-93G pelleted rodent diets

An extraction procedure (Qian, 1998) was used to qualitatively confirm the vitamin levels in both regular and vitamins A-and-D deficient AIN-93G pelleted rodent diet (Dyets, Inc., Bethlehem, PA). One gram of sample was precisely weighed, ground, and added to a screw-capped extraction tube. Four ml of acetone-chloroform solvent (30:70) mixture was added to the tube and the tube was flushed with N_2 to protect vitamins from air exposure before sealing with the cap. The mixture was shaken on a vortex mixer for 1 min, rested for 5 min, and mixed another minute. After centrifugation for 5 min, 1 ml of supernatant was transferred to a 1.5 ml vial and evaporated under nitrogen to remove the solvent. The residue was re-dissolved in hexane and assayed by normal phase HPLC. Chromatograms showed the presence of a RP peak in positive diet (Fig. 5.1) and no visible RP peak in deficient diet (data

not shown). RP retention time: 3.65 min. The peak at 5.62 min was probably the solvent peak of acetone. Vitamin D_3 extraction was also performed using the same method, however, due to presence of many interfering lipid compounds, vitamin D_3 peak was not separated from other peaks.

5.3.2.2 Body weight, weight gain, food and milk consumption

During depletion, marginal vitamin A deficiency did not hinder the rats from fast growth (Table 5.2). Normal growth and good health had been reported in another vitamin A deficiency study in rats (Lewis, 1990). For the 2-week repletion rats, there was no significant difference in final body weight, weight gain and milk consumption among all treatment groups, but lower food intake and liver weights were seen in the **N/N** and **N/O** groups than in the **N/P** and **N/N/O** groups. However, in the 4-week repletion rats, no difference was detected in either liver weight or food consumption among groups (Tables 5.2 –5.4).

Table 5.2 Body weight, liver weight and food consumption at baseline and depletion

Parameters/groups	Baseline	2-week depletion	4-week depletion
Final body weight (g)	[1] 47.6 ± 8.62	117.0 ± 9.44	280.5 ± 27.8
Daily weight gain (g)	N/A	5.9 ± 0.94	8.9 ± 1.1
Liver weight (g)	1.73 ± 0.51	7.13 ± 0.75	14.52 ± 0.75
Food intake (g/day)	N/A	12.4 ± 1.4	12.3 ± 1.5

[1]Data were shown as mean ± SD; N/A: data are not available.

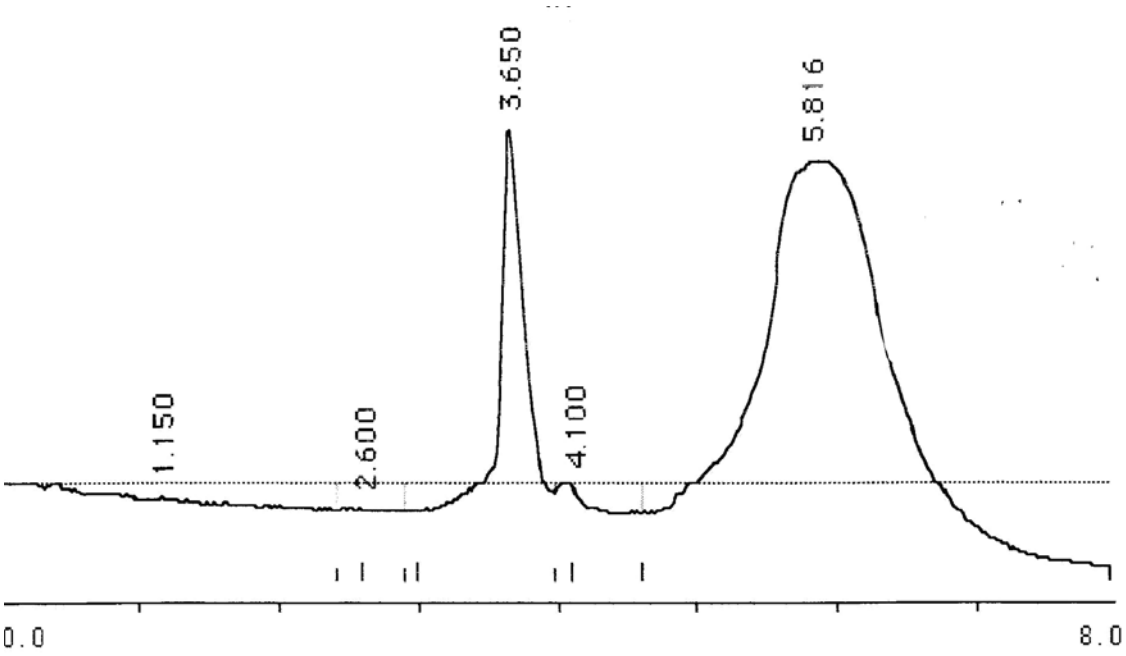

Figure 5.1 HPLC chromatograms of retinyl palmitate extracted from regular AIN-93G pellet rodent diet

Table 5.3 Body weight, liver weight and food and milk consumption during 2-week repletion

Parameters/ groups	P/N	N/N	N/P	N/O	N/N/O
Final body weight (g)	360.9 ± 16.8	352 ± 38.4	376.0 ± 36.7	367.2 ± 19.3	369.2 ± 10.7
Daily weight gain (g/day)	[1] 7.5 ± 0.5	7.4 ± 1.3	7.4 ± 1.4	8.3 ± 1.4	7.7 ± 0.6
Liver weight (g)	16.10 ± 3.22 [a, b]	14.65 ± 2.37 [b]	17.42 ± 1.93 [a]	14.71 ± 0.68 [b]	16.7 ± 2.76 [a]
Food intake (g/day)	22.6 ± 1.4 [a]	19.2 ± 4.3 [b]	22.3 ± 2.6 [a]	21.0 ± 2.6 [a, b]	22.4 ± 1.0 [a]
Milk consumption (g/day)	44.3 ± 4.5	46.8 ± 4.8	48.6 ± 3.0	40.9 ± 6.2	39.3 ± 5.8

[1]Data were shown as mean ± SD; means in a row without common superscripts letters are significantly different ($p<0.05$). N = 4 in the **P/N** and **N/N** groups. N= 5 in the other groups.

Table 5.4 Body weight, liver weight and food and milk consumption during 4-week repletion

Parameters/ groups	P/N	N/N	N/P	N/O	N/N/O
Final body weight (g)	[1] 413 ± 13.6	390.5 ± 34.5	413.4 ± 37.6	418.6 ± 14.7	416.7 ± 23.4
Daily weight gain (g)	6.9 ± 0.7	6.1 ± 0.4	6.2 ± 0.4	6.3 ± 0.6	6.0 ± 0.5
Liver weight (g)	16.32 ± 1.10	14.71 ± 0.93	15.55 ± 2.71	15.77 ± 2.19	15.54 ± 1.5
Food intake (g/day)	20.3 ± 2.0	19.1 ± 2.3	20.4 ± 2.2	20.1 ± 1.1	19.7 ± 1.1
Milk consumption (g/day)	45.4 ± 10.6	46.6 ± 5.6	47.8 ± 11.5	53.2 ± 8.7	50.0 ± 9.5

[1]Data were shown as mean ± SD; N = 4 in the **P/N** and **N/N** groups. N= 5 in the other groups.

5.3.3 Vitamins and mineral intake

According to the nutrient composition of AIN-93G (Reeves, 1993), one kg of the AIN-93G diet contains 4000 IU (2200 μg) of all-trans retinyl palmitate, 1000 IU (25 μg) of vitamin D_3, and 5000 mg calcium. Eight oz (240 ml) skim milk is reported to contain 302 mg calcium (Wardlaw, 2002). Based on the above information together with data from the UHT processed skim milk shelf-life study and rats' food and milk consumption, we were able to estimate the average intakes of vitamin A, vitamin D_3 and calcium in the rats during the bioavailability study (Tables 5.5- 5.9; Fig. 5.2-5.5).

Table 5.5 Vitamin A intake (µg /day/rat) as retinyl palmitate during repletion

Time / Group	P/N	N/P	N/O	N/N/O
2-week repletion	[1] 49.72 ± 3.08 [a]	10.21 ± 0.63 [c]	35.17 ± 5.33 [b]	11.27 ± 1.66 [c]
4-week repletion	44.66 ± 4.40 [a]	10.04 ± 1.73 [b]	37.24 ± 6.09 [a]	11.67 ± 2.22 [b]

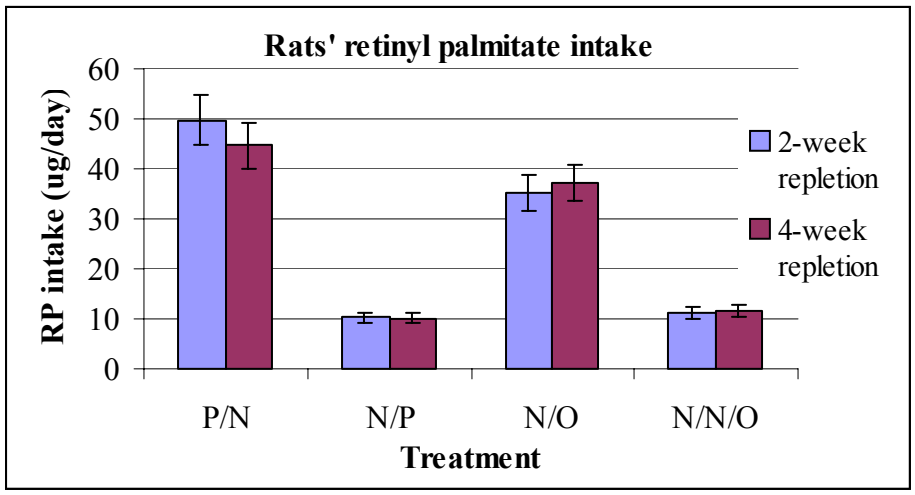

Figure 5.2 Retinyl palmitate intake during repletion

Table 5.6 Vitamin D intake (ng /day/rat) as vitamin D_3 during repletion

Time / Group	P/N	N/P	N/O	N/N/O
2-week repletion	[1] 565 ± 35 [b]	709.6 ± 43.8 [a]	433.5 ± 65.7 [c]	138.9 ± 20.5 [d]
4-week repletion	507.5 ± 50 [a, b]	616.6 ± 148.4 [a]	393.7 ± 64.4 [b]	123.3 ± 23.4 [c]

[1]Each value is the mean ± SD. N= 4 in the **P/N** group. N = 5 in the other groups. Means in a row without common superscripts letters are significantly different ($p<0.05$)

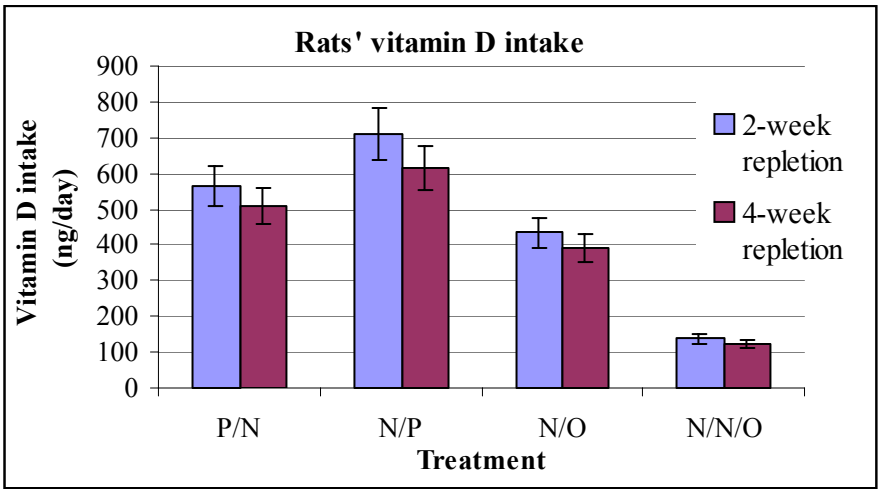

Figure 5.3 Vitamin D intake during repletion

Table 5.7. Calcium intake (mg/day/rat) from pelleted diet during depletion

Source/Group	2-week depletion	4-week depletion
AIN-93 diet	[1] 62.0 ± 7.0	61.5 ± 7.5

Table 5.8 Calcium intake (mg/day/rat) from pelleted diet and milk during 2-week repletion

Source /Group	P/N	N/N	N/P	N/O	N/N/O
AIN-93 diet	[1] 113 ± 7.0	96 ± 21.5	111.5 ± 24.2	105.0 ± 13.0	112.0 ± 5.0
Skim milk	412.0 ± 41.9	435.2 ± 44.6	452.0 ± 27.9	380.4 ± 57.7	365.3 ± 53.9

[1]Each value is the mean ± SD. N= 4 in the **P/N** group. N = 5 in the other groups.

Table 5.9 Calcium intake (mg/day/rat) from pelleted diet and milk during 4-week repletion

Source /Group	P/N	N/N	N/P	N/O	N/N/O
AIN-93 diet [1]	101.5 ± 10.0	95.5 ± 11.5	102 ± 11.0	100.5 ± 5.5	98.5 ± 5.5
Skim milk	422.2 ± 98.6	433.4 ± 52.1	444.6 ± 107.0	494.8 ± 80.9	465.0 ± 88.4

[1]Each value is the mean ± SD. N= 4 in the **P/N** and **N/N** groups, n = 5 in the other groups.

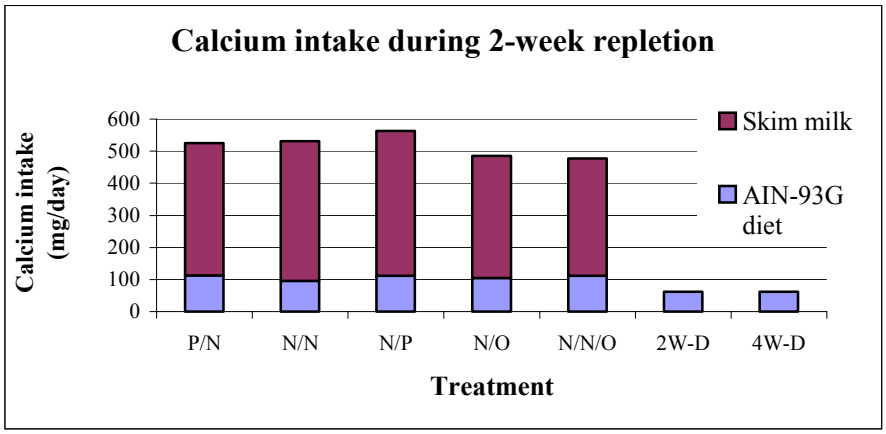

Figure 5.4 Calcium intake during depletion and 2-week repletion

2W-D: 2-week depletion; 4W-D: 4-week depletion

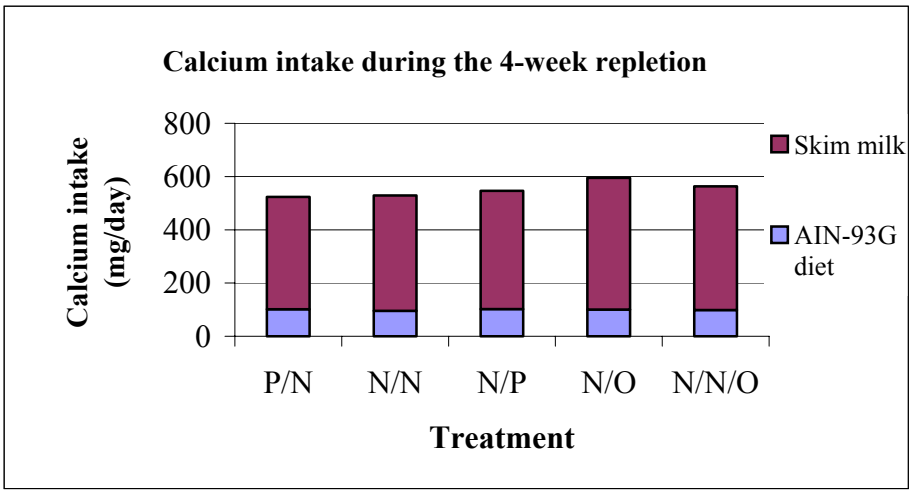

Figure 5.5 Calcium intake during the 4-week repletion

5.3.4 Assay for serum 25-OH vitamin D_3

25-OH vitamin D ^{125}I RIA kit (DiaSorin Co., Stillwater, MN)) had been shown to exhibit excellent agreement with HPLC methods and accurately estimate total circulating 25-OH vitamin D (25-OH D), including both 25-OH D_2 and 25-OH D_3 (Hollis, 2000). The RIA kit was used to indicate vitamin D status in the rats.

5.3.4.1 Materials and Method

Blood was collected by cardiac puncture into a 4 ml vacutainer (coated with clot activator). The blood was allowed to clot at room temperature for 30 min and centrifuged for 15 min at 760 × *g* to obtain sera with no evidence of hemolysis. The serum was stored in amber sample vials at -20°C. The extraction used labeled 12×75 mm disposable borosilicate glass tubes for each standard, control and samples. Acetonitrile (500 µl) was added to each tube by placing the pipet tip containing 50 µl of standard, control or sample below the surface of the acetonitrile and SLOWLY adding into the acetonitrile, followed by vortexing 10 sec. The mixture was centrifuged at 1200 × *g* for 10 min at 20-25°C. Duplicate 25-µl aliquots from the supernatant were pippetted into separate appropriately labeled 12×75mm tubes. Prior to the assay, all reagents and samples were equilibrated to room temperature. Into labeled disposable tubes for total counts, non-specific binding (NSB), standards, controls, and unknown samples were added 25 µl each of extracted standards, controls and samples. Fifty µl of ^{125}I - labeled 25-OH-D radioactive tracer was added to each tube. One ml NSB/addition buffer was added to total counts and NSB tubes. One ml 25-OH-D antiserum was added to each tube except for total counts and NSB. After thorough mixing and 90 min

incubation at 20-25°C, 500 µl DAG precipitating complex was added to all the tubes except for the total counts tube. Tubes were mixed well and incubated for 25 min at 20-25°C. Then NSB/addition buffer was added to all the tubes except for total counts and NSB. Samples were centrifuged at 1800 × *g* for 20 min. The supernatants were decanted and each tube was counted in the gamma counter (Cobra II Auto Gamma Counter 101499, Packard Inc. Norwalk, CT) for 60 sec or longer. Standard curves were obtained by plotting the extent of binding against stated concentrations of the calibration standards on a linear or logarithmic scale (Fig. 5.6).

5.3.4.2 Results

After the 2-week depletion, serum 25-OH D in rats dropped from the baseline level of 22.96 ng/ml to zero as calculated from the standard curve. Another 2-week depletion further ensured the vitamin D deficiency (Table 5.10). The 2-week repletion of the vitamin significantly boosted the serum 25-OH D level in all treatment groups except for the **N/N** group (Table 5.11), with higher vitamin levels in the groups **P/N** and **N/P** than the groups **N/O** and **N/N/O**. During the 4-week repletion, serum vitamin D was even higher in all the groups other than the **N/N** group (Table 5.11), with the highest level in the **N/P** group followed by the **P/N** and **N/O** groups.

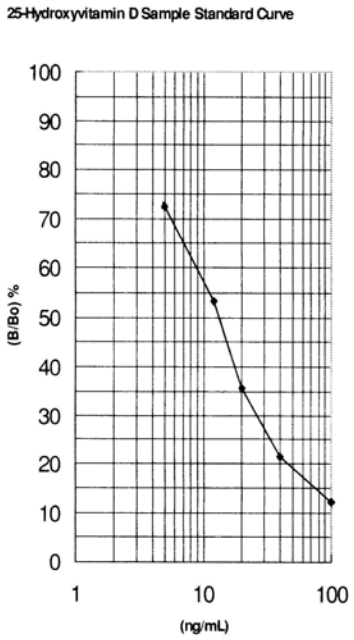

Figure 5.6 Sample standard curve of serum 25-OH vitamin D

Table 5.10. Serum 25-OH D concentration (ng/ml) at baseline and depletion

	Baseline	2-week depletion	4-week depletion
Serum 25-OH D	[1] 22.96 ± 2.97[a]	0 [b]	0 [b]

Table 5.11. Serum 25-OH D concentration (ng/ml) after 2-week and 4-week repletion

Time/ Group	P/N	N/N	N/P	N/O	N/N/O
2-week repletion	[1] 16.56 ± 0.21[a]	0 [d]	18.62 ± 1.75 [a]	14.56 ± 2.63 [b]	7.86 ± 2.02 [c]
4-week repletion	17.01 ± 1.83 [b]	0 [d]	21.42 ± 4.72 [a]	17.06 ± 5.37 [b]	9.21 ± 3.72 [c]

[1]Each value is the mean ± SD. Means in a row without common superscript letters are significantly different ($p<0.05$). N= 4 in the **P/N** and **N/N** groups, n = 5 in the other groups.

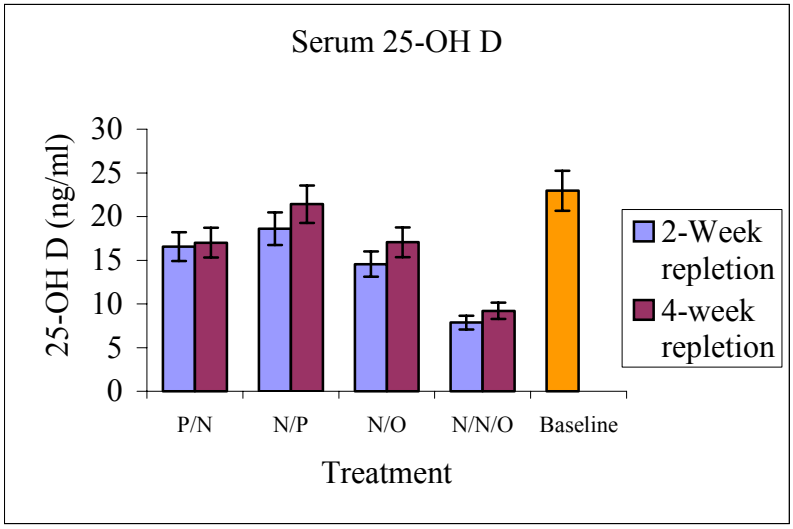

Figure 5.7 Comparison of serum 25-OH D concentrations

5.3.4.3 Discussion and Conclusion

As vitamin D storage in weanling rats is very low, vitamin D deficiency was developed right after the 2-week depletion as indicated by undetectable level of serum 25-OH D, which was significantly different from the baseline data. The progress of vitamin D deficiency was consistent with other studies (Kollenkirchen, 1991 and Toromanoff, 1997). However, as complete depletion of vitamin D is difficult to achieve, serum 1, 25-$(OH)_2$ D might still be present, which is a relatively common feature in studies of vitamin D-deficient animals (Walters, 1992). No serum 25-OH D detected in the rats of the negative control group suggested neither vitamin D deficient diet nor non-fortified skim milk was adequate to replete the vitamin. As expected, during the 4-week repletion, rats in all the treatment groups other than the negative control group had their serum 25-OH vitamin D increased to different extents. As addressed in section 4.4.1, skim milk was fortified with BLG-complexed and oil-based vitamin D to the same level to meet the vitamin D requirement of rats as AIN-93G pelleted diet does. Protein-complexed vitamin fortified skim milk (PFM) exhibited the

highest stability of vitamin D_3 during the UHT processed milk shelf life study (Table 4.11 and Fig.4.10) and boosted the serum 25-OH D more than the other treatments (Table 5.11). Though the vitamin D level dropped to half the targeted level in oil-based vitamin fortified skim milk (OFM) at the end of the 6-week shelf life (Table 4.11and Fig 4.10), it still raised the serum 25-OH D to the extent equivalent to regular AIN-93G. It is a positive dose response that the rats in the **N/N/O** group exhibit the least increase in serum 25-OH D among groups and serum 25-OH D was higher after the 4 weeks repletion than after the 2 weeks repletion in all treatment groups except for the **N/N** groups which did not differ. This experiment was intended to compare the bioavailability of vitamin D in OFM and PFM, but due to difference in shelf life stability and actual vitamin intake, it is difficult to make such a comparison. However, we can conclude that vitamin D was more stable in PFM than in OFM and the targeted level was adequate to replete and maintain normal serum vitamin D level in the rats. The reason that vitamin D positive diet was not as efficient may be due to degradation and less bioavailability of the vitamin. For future bioavailability studies, degradation of vitamins in diet or milk should be carefully monitored and prevented.

5.3.5 Assays for bone and serum calcium

5.3.5.1 Methods

Serum calcium (Slavin, 1988)

Blood samples were centrifuged at 3500 rpm for 15 min at room temperature using a Microfuge R centrifuge (Roche Biomedical Laboratories, Inc. Burlington, NC). Serum calcium level was determined using atomic absorption spectrophotometry (Perkin Elmer 3100, Norwalk, CT). Serum samples were diluted 1: 50 with 0.5% lanthanum in 0.1 N HCl.

The presence of lanthanum controls chemical interference from phosphate when determining calcium. A 0.5% lanthanum in 0.1N HCl solution was also used as a blank. Calcium standard solutions (1, 3, 5, 7, and 9 ppm) were made from the 1000 ppm calcium stock solution (Fisher, Pittsburgh, PA).

Femur weight and ash percentage (Banu, 1999)

Femurs were cleaned of adhering tissue and placed in 100% ethanol overnight. The solvent was removed and bones were soaked in chloroform overnight. The bones were removed from the chloroform and allowed to air-dry for 5min. Bones were placed in individual crucibles that had been baked in a muffle furnace, then cooled in a desiccators for 3 hr and weighed. The bones were then baked at 100°C for 8-12 hr. Upon removal from the 100°C oven, the bones were placed in the dessicator to cool. After 3 hr, the bones were weighed. This measurement was termed the dry weight. After weighing, the bones were placed in a muffle furnace and ashed at 649°C for 24 hr. Ash weight was determined. Bone ash was pre-dissolved in 3 N HCl and then diluted 1:1000 using 0.5% lanthanum in 0.1 N HCl. All glassware was acid-washed with 1 N HCl before using. Each sample was read in duplicate on the atomic absorption spectrometer (Perkin Elmer Model 3100, Norwalk, CT).

5.3.5.2 Results

There was a significant difference in femur weight and serum protein concentrations between baseline and depletion groups due to age. Calcium percentage in bone ash did not differ among groups. Serum calcium started to drop during the 2-week depletion and decreased more during 4-week depletion (Table 5.12). During the 2-week repletion, femur dry weight was significantly lower in the **N/N** group than the **N/P** group. Serum protein and

calcium levels were lower in the **N/O** group. There was no significant difference detected in bone ash percentage and calcium percentage (Table. 5.13).

Table 5.12 Bone and serum mineral characteristics at baseline and during depletion

Parameters/groups	Baseline	2-week depletion	4-week depletion
Femur dry weight (mg)	[1] 58.24 ± 19.32	195.88 ±13.43	379.74 ± 28.95
Bone Ash percentage, %	47.39 ± 2.49	53.48 ± 2.77	57.88 ± 1.10
mg Ca/mg ash	0.39 ± 0.02	0.38 ± 0.01	0.38 ± 0.01
Serum protein (mg/100ml)	4.36 ± 0.11	5.36 ± 0.18	6.06 ± 0.29
Serum calcium (mg/L)	131.31 ± 6.41 [a]	118.45 ± 28.71 [b]	107.5 ± 9.71 [c]

During 4-week repletion, femur dry weight was lower in the **N/N** group than the other groups. But no significant difference was detected in the other parameters among all treatment groups (Table 5.14).

Table 5.13. Bone and serum mineral characteristics after 2-week repletion

Parameter /group	P/N	N/N	N/P	N/O	N/N/O
Femur dry weight (mg)	**524.65 ± 38.74** [a, b]	**504.48 ± 32.83** [b]	**556.64 ± 36.45** [a]	**542.80 ± 67.73** [a, b]	**544.9 ± 28.24** [a, b]
Bone Ash percentage, %	[1] 58.65 ±1.13	59.07 ± 0.87	57.89 ± 0.42	59.54 ± 2.58	58.77 ± 0.76
mg Ca/mg ash	**0.39 ± 0.01**	**0.39 ± 0.01**	**0.38 ± 0.03**	**0.40 ± 0.01**	**0.41 ± 0.02**
Serum protein (mg/100ml)	6.64 ± 0.70 [a]	6.43 ± 0.48 [a]	6.42 ± 0.22 [a]	5.82 ± 0.61 [b]	6.30 ± 0.87 [a, b]
Serum calcium (mg/l)	**128.13 ± 31.32** [a, b]	**138.44 ± 12.49** [a]	**142.80 ± 24.30** [a]	**115.75 ± 15.19** [b]	**143.90 ± 12.95** [a]

[1]Each value is the mean ± SD and n = 4 in negative and positive control groups, and n=5 in the other groups. Means in a row without common superscript letters are significantly different ($p<0.05$).

Table 5.14. Bone and serum mineral characteristics after 4-week repletion

Parameter /group	P/N	N/N	N/P	N/O	N/N/O
Femur dry weight (mg)	**595.60 ± 32.34** [a, b]	**574.28 ± 37.92** [b]	**600.24 ± 31.10** [a, b]	**630.96 ± 10.30** [a]	**632.26 ± 29.71** [a]
Bone Ash percentage, %	[1] 61.76 ± 0.48	61.87 ± 1.32	62.36 ± 0.78	61.02 ±2.31	61.52 ± 0.17
mg Ca/mg ash	**0.41 ± 0.01**	**0.39 ± 0.01**	**0.40 ± 0.03**	**0.39 ± 0.01**	**0.42 ± 0.02**
Serum protein (mg/100ml)	6.53 ± 0.10	6.23 ± 0.23	6.23 ± 0.27	6.2 ± 0.32	6.62 ± 0.35
Serum calcium (mg/L)	**119.81 ± 16.64**	**131.44 ± 11.93**	**136.30 ± 19.57**	**125.70 ± 17.00**	**136.55 ± 2.75**

[1]Each value is the mean ± SD and n = 4 in negative and positive control groups, and n=5 in the other groups. Means in a row without common superscript letters are significantly different ($p<0.05$).

5.3.5.3 Discussion and Conclusion

Despite progressing vitamin D deficiency, the rats still showed fast bone growth during depletion, though lower serum calcium was detected in the deficient animals (Table 5.12). During repletion, it was unexpected that there was no significant difference in bone ash percentage, calcium percentage in ash, and serum calcium among groups, though the serum 25-OH D assay suggested vitamin D deficiency in the rats from the **N/N** group throughout the study. The possible explanations include: 1. More than adequate dietary sources of calcium from AIN-93G rodent diet and skim milk might stimulate calcium absorption and utilization through vitamin D independent pathways, resulting in normal bone and serum calcium characteristics even with vitamin D deficiency. Other studies also reported normal or slightly sub-normal bones in vitamin D-deficient rats when the mineral supply is adequate (Toromanoff, 1997 and Kollenkirchen, 1991). Further, calcium absorption through passive paracellular pathway may have been enhanced by lactose in the milk (Bronner, 2003). 2. Due to needs for fast growth, young rats absorb calcium more efficiently through passive

diffusion. 3. The eight weeks of vitamin D deficiency may not be enough time for older rats to develop and show visible symptoms, though younger rats were more susceptible to the deficiency. We cannot rule out the hypothesis that the remaining 1, 25-$(OH)_2$ D with the intestinal up-regulation of the vitamin D receptor may be responsible for the normal serum calcium observed. However, high calcium diet and vitamin D deficiency were reported to impair the synthesis of VDR (Kollenkirchen, 1991). In this study, calcium from AIN-93G pelleted diet alone could not maintain normal serum calcium under vitamin D deficiency (Table. 5.12), but calcium with lactose from skim milk helped maintain normal serum calcium and bone mineralization even in vitamin D deficient animals during repletion (Tables 5.13 and 5.14). These results indicate that vitamin D does not play an important role in the actual mineralization process, and the failure to mineralize the bone in vitamin D deficiency is due to inadequate levels of calcium and phosphorus in the plasma.

The results of this experiment agree with recent findings from vitamin-D receptor (VDR) knockout mice (Li, 1997; Yoshizawa, 1997). The mutant mice had no defects in development and growth before weaning, but bone formation and mineralization were severely impaired after weaning, which suggested that calcium content in the milk offset the effect of vitamin D deficiency. When VDR knockout mice were maintained on a rescue diet containing high calcium, the impaired bone mineralization and formation were recovered (Amling, 1999). This confirmed that the direct role of 1, 25-$(OH)_2$ D in bone is less important than its action on the intestine. It is likely that vitamin D is not needed for bone calcium but is responsible for maintaining extracellular calcium and phosphorus concentrations in a supersaturated state when dietary calcium is low, which results in mineralization of bone.

5.3.6 Assay for bone rheology

5.3.6.1 Method (Raab, 1990)

The mechanical properties of the left femur were determined using a three-point bending test with an Instron Universal Testing machine. The left femur was removed from the animals, cleaned of all soft tissue, individually sealed in plastic bags and frozen at -20°C. While in the sealed bags, the bones were thawed to room temperature on the day of testing. The freeze-thaw procedures should not alter the mechanical properties of bone (Seldin, 1965). In the three-point bending test, the bones were rested on two support plates, 1.94 cm apart. Force was applied at the midpoint of the bone by a plunger moving downwards at a constant rate of 5 mm/min. A typical load-deformation curve during three-point bending test is shown in Fig.5.8. Results are shown in Tables 5.15 and 5.16. Among the parameters, stress is the force withstood per unit area of bone and strain is a measure of the bending of the bone relative to its length.

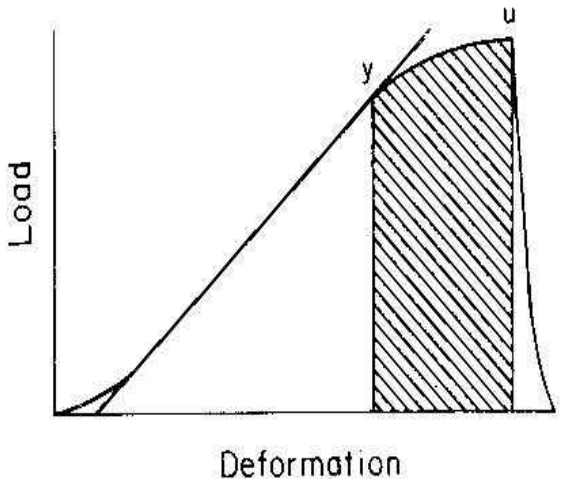

Figure 5.8. Typical load-deformation curve during a three-point bending test

y, yield or bending point; u, ultimate or breaking point; horizontal axis, vertical deformation of the bone as plunger moved downward at a constant rate; vertical axis, load on bone or force with which bone resisted deformation. White area, elastic region where no permanent damage occurs to bone; hatched area, plastic region where bone is permanently damaged.

Table 5.15 Bone biomechanical characteristics by three-point bending study after 2-week repletion

Parameters/ Groups	P/N	N/N	N/P	N/O	N/N/O
Peak load (gm)	[1] 8814 ± 140	8899 ± 526	8500 ± 759	8811 ± 527	8813 ± 504
Deformation (mm)	1.10 ± 0.30	1.42 ± 0.42	1.27 ± 0.30	1.27 ± 0.24	1.26 ± 0.11
Compression Strain	0.32 ± 0.10	0.45 ± 0.17	0.39 ± 0.11	0.38 ± 0.10	0.38 ± 0.04
Tension Strain	0.16 ± 0.05	0.23 ± 0.08	0.19 ± 0.06	0.19 ± 0.05	0.19 ± 0.02
Shear Strain	0.49 ± 0.16	0.67 ± 0.25	0.58 ± 0.17	0.58 ± 0.14	0.57 ± 0.06
Compression Stress (kPa)	3198 ± 379	2873 ± 567	2879 ± 212	3002 ± 278	3016 ± 273
Tension Stress (kPa)	1599 ± 190	1436 ± 283	1439 ± 106	1501 ± 139	1508 ± 137
Shear Stress (kPa)	2398 ± 284	2155 ± 425	2159 ± 159	2251 ± 209	2262 ± 205

Table 5.16 Bone biomechanical characteristics by three-point bending study after 4-week repletion

Parameter /group	P/N	N/N	N/P	N/O	N/N/O
Peak load (gm)	[1] 10393 ± 86	9770 ± 1328	9813 ± 297	10892 ± 881	10181 ± 384
Deformation (mm)	0.94 ± 0.08	1.03 ± 0.11	1.03 ± 0.18	1.05 ± 0.11	1.07 ± 0.13
Compression Strain	0.27 ± 0.03	0.30 ± 0.04	0.30 ± 0.06	0.30 ± 0.04	0.31 ± 0.04
Tension Strain	0.13 ± 0.01	0.15 ± 0.02	0.15 ± 0.03	0.15 ± 0.02	0.16 ± 0.02
Shear Strain	0.40 ± 0.04	0.57 ± 0.06	0.45 ± 0.09	0.46 ± 0.06	0.47 ± 0.07
Compression Stress (kPa)	3972 ± 134	3610 ± 398	3647 ± 311	4024 ± 448	3718 ± 142
Tension Stress (kPa)	1987 ± 67	1805 ± 199	1824 ± 155	2012 ± 224	1859 ± 71
Shear Stress (kPa)	2979 ± 100	2707 ± 298	2735 ± 233	3018 ± 336	2788 ± 107

[1]Each value is the mean ± SD and n = 4 in the P/N and N/N groups, and n=5 in the other groups.

5.3.6.2 Results and Discussion

Data showed no significant difference for any parameters among treatment groups at 2-week or 4-week repletion, but there was a dramatic difference between 2-week repletion and 4-week repletion within individual groups probably due to the age difference (Tables 5.15 and 5.16). In conclusion, vitamin D deficiency did not affect bone rheology, probably due to an over-adequate consumption of dietary calcium from AIN-93G rodent diet and skim milk.

5.3.7 Assays for serum retinol, liver retinol and retinyl palmitate

5.3.7.1 Serum and liver samples preparation

In a disposable glass centrifuge tube, 200 μl ethanol (0.01%BHT) was added into 200 μl serum, and 0.1 μg retinyl acetate in 100 μl ethanol was added as the internal standard. Then 2 ml hexane was added, and the tubes were capped and vortexed vigorously for 1.5min. After adding 100 μl water, the tubes were vortexed for another 1.5 min. Then they were centrifuged for 5 min to separate phases. The upper hexane layer was transferred to amber sample vials for HPLC analyses.

Whole rat liver was weighed, cut into pieces with scissors and homogenized with an equal weight of distilled water for 2.5 min. One gram of the liver homogenate was pipetted into a centrifuge tube. Two ml of 0.01% BHT ethanol, 50 μl of 1mg/ml retinyl acetate (internal standard), and 2 ml of hexane were added into the tube. The tube was capped and vortex-mixed vigorously for 1.5 min. The tube was uncapped and another 2 ml of hexane was added and vortex-mixed vigorously for another 1 min. A 500-μl volume of distilled

water was added and vortexed for 10 sec. The tube was centrifuged for 5 min. The upper hexane layer was transferred to an amber sample vial for HPLC analyses.

5.3.7.2 HPLC analyses for liver and serum vitamin A

To better separate retinol and retinyl acetate in normal-phase HPLC, a mobile phase other than that described in Chapter 4 was used (Ruotolo, 1992). Most published procedures for simultaneous analysis of polar retinoids, such as retinol, and nonpolar retinoids, such as retinyl palmitate, have used reversed-phase HPLC and solvent gradient. This results in much longer time for analysis and equilibration between analyses. The method described by Ruotolo and coworkers successfully allowed separation of retinol and retinyl acetate (as internal standard) on a normal phase column with an isocratic mobile phase (hexane: n-butyl chloride: acetonitrile 82:13:5, with 0.01 ml of acetic acid). To further shorten the running time and improve the separation of retinol, a flow rate of 1.5 ml/min instead of 1 ml/min was used and 0.1 ml acetic acid was added into 100 ml mobile phase.

Tubes for standard curves and recovery study were prepared according to tables 5.17 and 5.18. For a standard curve, the vitamin stock solutions were spiked accurately into 4 separate tubes and the solvents were evaporated under nitrogen until dry. Hexane was added to each tube afterwards and the tubes were vortexed before HPLC injection. For the recovery study, the stock solutions were accurately spiked into 4 tubes containing 200 μl serum or one gram liver homogenate. Vitamin A was extracted according to the protocol described in section 5.3.7.1. Hexane layers were filtered and used for HPLC analysis. Tubes without spikes served as control. HPLC analyses were performed as described in section 4.2 except for the mobile phase. AUC of retinol (ROH), retinyl acetate (RA) and retinyl palmitate (RP) were

recorded and standard curves of serum and liver vitamin A were plotted as in Fig.5.9-5.11. The recovery was calculated as AUC in the recovery study tubes divided by that in the corresponding standard curve tube (Tables 5.19 and 5.20). Typical HPLC chromatograms for liver and serum vitamin A samples are shown in Fig.5.12 and 5.13. The retention times for RP, RA and ROH are 2.00, 2.88, and 7.75 min.

Table 5.17 Preparation of standard curve for serum samples

Sample #	**ROH** (ng)	**RA** (ng)	**ROH AUC**	**RA AUC**	**ROH/RA**	**ROH** (μg/ml serum)
#1	50	100	58677	116517	0.50359	0.25
#2	150	100	179960	124796	1.44203	0.75
#3	250	100	321742	129924	2.47639	1.25
#4	350	100	434322	119397	3.63763	1.75

Table 5.18 Preparation of standard curve for liver samples

#	**ROH** (μg)	**RA** (μg)	**RP** (μg)	**ROH AUC**	**RA AUC**	**RP AUC**	**ROH /RA**	**RP /RA**	**ROH** (μg/g liver)	**RP** (μg/g liver)
#1	**2**	**5**	**25**	179777	526308	1411558	0.34	2.68	4	50
#2	**4**	**5**	**50**	337906	493242	3113389	0.69	6.31	8	100
#3	**6**	**5**	**75**	558505	521009	4523414	1.07	8.68	12	150
#4	**8**	**5**	**100**	809154	545983	6418235	1.48	11.76	16	200

Table 5.19 The recovery of serum vitamin A

#	RA AUC in recovery study	RA AUC in Standard curve	RA Recovery	ROH AUC in Recovery study	ROH AUC in Standard curve	ROH Recovery
1	122600	116517	1.05	51558	58677	0.88
2	124051	124796	0.99	176903	179960	0.98
3	116991	129924	0.90	274409	321742	0.85
4	130284	119397	1.09	528209	434322	1.21

The average recovery rate is RA 101%; The average recovery rate of ROH is 98%.

Table 5.20 The recovery of liver vitamin A

#	RA AUC in Recovery study	RA AUC in Standard Curve	RA Recovery	ROH AUC in Recovery study	ROH AUC in Standard curve	ROH Recovery	RP AUC in Recovery study	RP AUC in Standard curve	RP Recovery
1	515966	526308	0.98	174369	179777	0.97	1364158	1411558	0.97
2	534641	493242	1.08	348959	337906	1.03	3012243	3113389	0.97
3	505083	521009	0.97	474747	558505	0.85	4165941	4523414	0.92
4	553330	545983	1.01	681264	809154	0.84	6064351	6418235	0.95

The average recovery rates of RA, ROH and RP are 101%, 92% and 95%, respectively.

*AUC in recovery studies = AUC in sample –AUC in control.

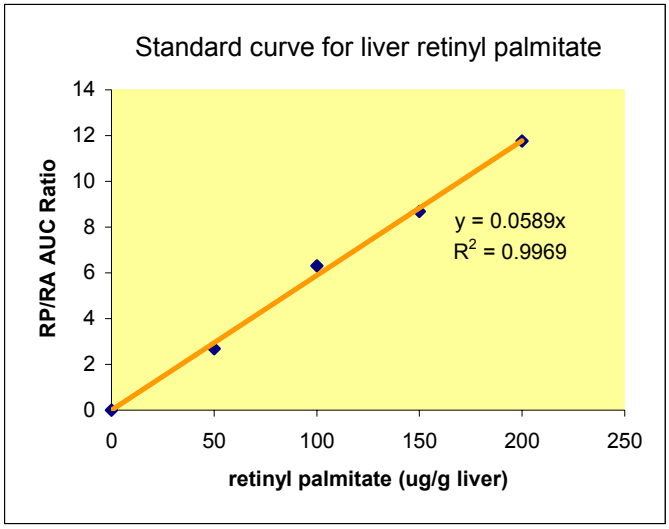

Figure 5.9. Standard curve for liver vitamin A palmitate

5.3.7.3 Results of vitamin A from rats' liver and serum samples

Serum retinol concentrations were not significantly different between baseline and depletion, however, vitamin A deficiency dramatically dropped the liver retinol and retinyl palmitate concentrations in depleted rats as compared to the baseline (Table 5.21 and Fig.5.14). After 2-week repletion, no significant difference in serum retinol was detected among treatment groups, but liver retinol and retinyl palmitate levels were significantly higher in Groups **P/N** and **N/O** than the other groups (Table 5.22). After the 4 weeks repletion, serum retinol in the **P/N** group was significantly higher than the **N/P** group and serum retinol in the **N/N** group was the lowest among all groups. Liver vitamin A at 4 weeks of repletion exhibited a similar pattern to that after the 2 weeks of repletion (Table 5.23, Fig.5.14 and 5.15).

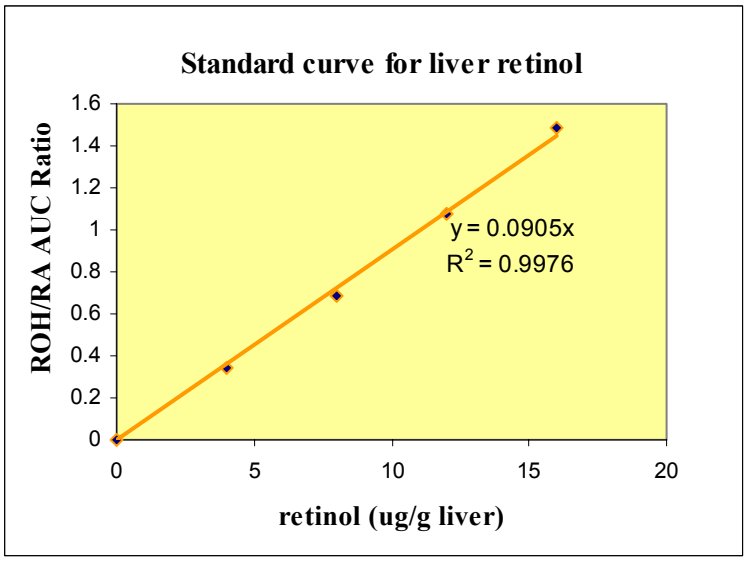

Figure 5.10 Standard curve for liver retinol

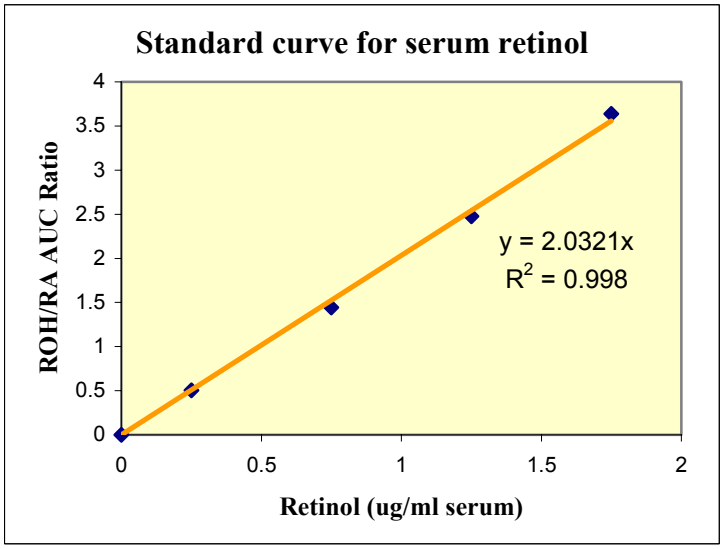

Figure 5.11 Standard curve for serum retinol

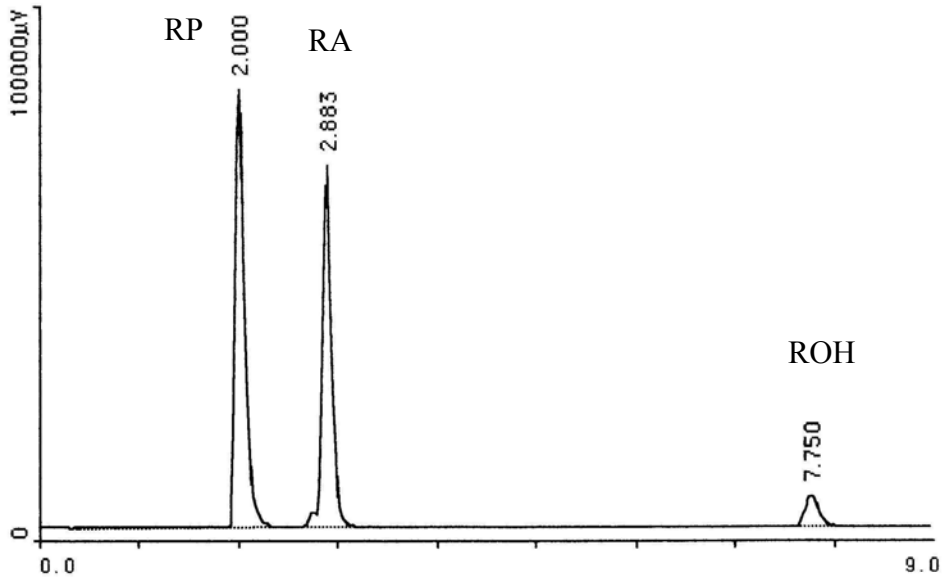

Figure 5.12. HPLC chromatogram of retinyl palmitate and retinol from the liver sample with retinyl acetate (internal standard)

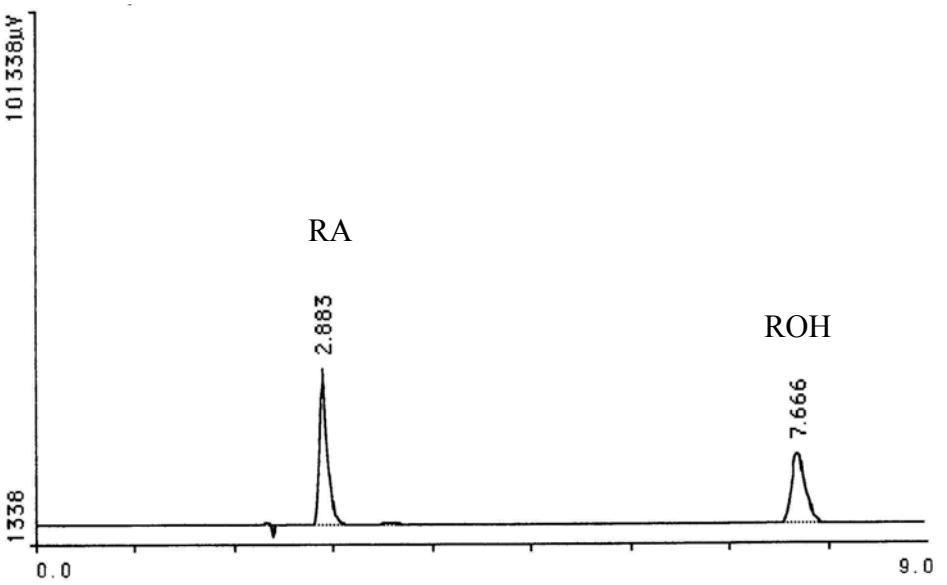

Figure 5.13 HPLC chromatogram of serum retinol with retinyl acetate (internal standard)

Table 5.21 Liver and serum vitamin A at baseline and during depletion

Parameters/Group	Baseline	2-week depletion	4-week depletion
Serum retinol (μg/ml)	[1] 0.29 ± 0.06	0.28 ± 0.07	0.31 ± 0.11
Liver retinol (μg/g)	4.95 ± 3.77 [a]	0.96 ± 0.28 [b]	0.32 ± 0.16 [c]
Liver retinyl palmitate (μg/g)	168.32 ± 46.00 [a]	30.99 ± 8.83 [b]	9.74 ± 4.26 [c]

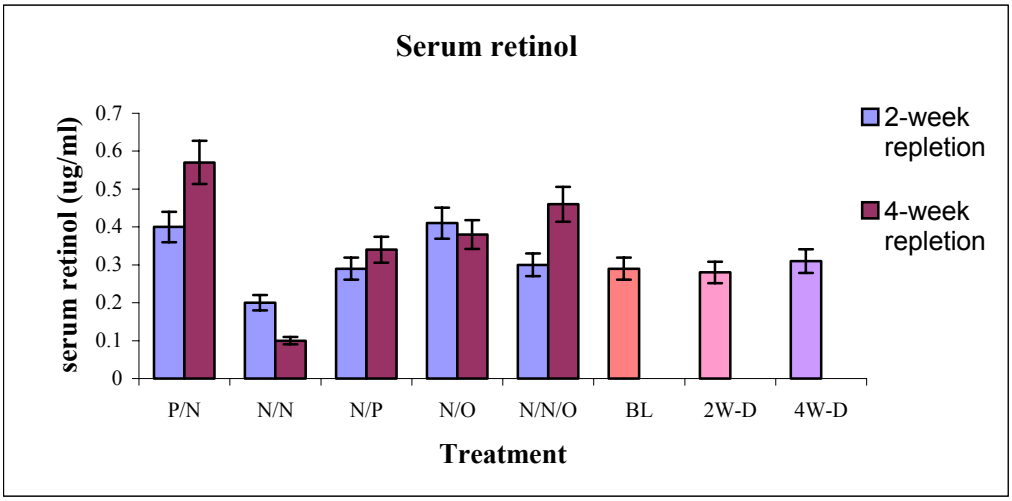

Figure 5.14 Comparison of serum retinol concentrations

BL: baseline; 2W-D: 2-week depletion; 4W-D: 4-week depletion.

Table 5.22 Liver and serum vitamin A after 2-week repletion

Parameters/ Group	P/N	N/N	N/P	N/O	N/N/O
Serum retinol (μg/ml)	[1] **0.40 ± 0.24**	**0.20 ± 0.08**	**0.29 ± 0.16**	**0.41 ± 0.23**	**0.30 ± 0.23**
Liver retinol (μg/g)	**1.14 ± 0.09** [a]	**0** [c]	**0.01 ± 0.02** [c]	**0.79 ± 0.53** [b]	**0.15 ± 0.21** [c]
Liver retinyl palmitate (μg/g)	**15.56 ± 3.46** [a]	**1.08 ± 0.46** [b]	**1.23 ± 0.80** [b]	**11.40 ± 4.76** [a]	**0.99 ± 0.69** [b]

[1]Data are shown as mean ± SD; means in a row without common superscripts letters are significantly different ($p<0.05$). N = 5.

Table 5.23 Liver and serum vitamin A after 4-week repletion

Parameters /Group	P/N	N/N	N/P	N/O	N/N/O
Serum retinol (μg/ml)	[1] 0.57 ± 0.10 [a]	0.10 ± 0.03 [c]	0.34 ± 0.13 [b]	0.38 ± 0.13 [a, b]	0.46 ± 0.15 [a, b]
Liver retinol (μg/g)	1.53 ± 0.28 [a]	0.11 ± 0.13 [b]	0.30 ± 0.21 [b]	0.84 ± 0.21 [a]	0.39 ± 0.30 [b]
Liver retinyl palmitate (μg/g)	36.09 ± 12.39 [a]	0.39 ± 0.15 [b]	1.30 ± 0.55 [b]	33.53 ± 8.79 [a]	4.88 ± 3.23 [b]

[1]Each value is the mean ± SD and n = 4 in negative and positive control groups, and n=5 in the other groups. Means in a row without common superscript letters are significantly different ($p<0.05$).

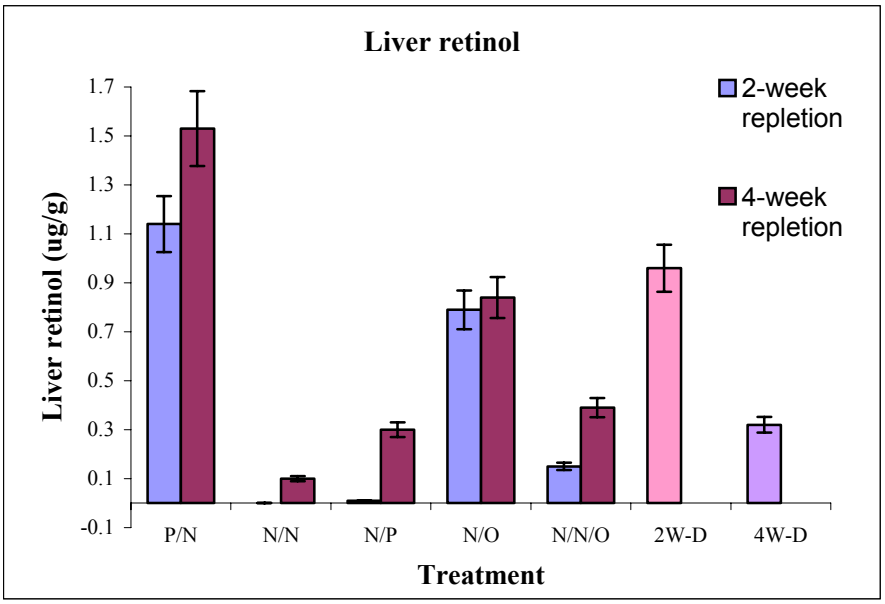

Figure 5.15 Comparison of liver retinol concentrations

2W-D: 2-week depletion group; 4W-D: 4-week depletion group.

5.3.7.4 Discussion and Conclusion

As addressed in the literature review, simultaneous analyses of retinyl esters and retinol usually have been performed on reversed-phase columns, requiring tedious sample preparation, solvent gradients and long assay times. In this study, a HPLC protocol developed by Ruotolo and co-workers in 1992 was modified and successfully resolved

retinyl palmitate, retinyl acetate (internal standard), and retinol during a less than 10-min run on the silica column using an isocratic mobile phase. Similar recovery rates were obtained for all the analytes. This method is efficient for the regular study of vitamin A in liver and serum samples.

Due to relatively small liver vitamin A storage in rats, vitamin-deficient diet successfully depleted vitamin A in the rats during the 4-week depletion (Table 5.21). The rats exhibited a normal progress of vitamin A deficiency: reduction of liver storage appeared first, followed by reduction in serum retinol. Weanling rats had been successfully used to develop rapid vitamin A deficiency by other studies (Gardner, 1993, Lewis, 1990, and Sivakumar, 1993). Lewis and co-workers (1990) provided descriptive evidence of an efficient metabolism of vitamin A from absorption through turnover and utilization in rats with very low vitamin A status. They found the deficient rats maintained a higher level of recycling of retinol between the plasma and the liver and other tissues, though their body stores of vitamin A were extremely low. This also explained the results in this study: when fed vitamin A deficient diet, the rats maintained normal serum retinol for 2 weeks while liver vitamin A storage dropped dramatically. During the repletion period, oil-based retinyl palmitate in skim milk repleted liver and serum vitamin A equivalent to vitamin A-containing AIN-93G pelleted rodent diet, indicating similar bioavailability of retinyl palmitate in skim milk and pelleted diet given the similar vitamin A intake in these two groups. But no treatment had brought liver vitamin A status back to normal by the end of the repletion due to the time limit (Table 5.22 and Table 5.23). As addressed in the shelf life study, the vitamin A level in UHT pasteurized PFM was about 1/3 of the targeted fortification level and continued dropping to 1/5 of the level in OFM. PFM still boosted the serum retinol to the level equivalent to that in

the **N/O** group, suggesting retinyl palmitate in PFM was adequate to maintain normal serum retinol level in the rats. The **N/N/O** group was designed to provide the equivalent retinyl palmitate to PFM and the vitamin A intake between the two groups was similar (Table 5.5). As expected, no significant difference was detected in liver vitamin A between the **N/N/O, N/P** and **N/N** groups, though the levels were higher in the first two groups. In summary, there was no significant difference in bioavailability of retinyl palmitate between PFM and OFM based on data from liver and serum vitamin A assays.

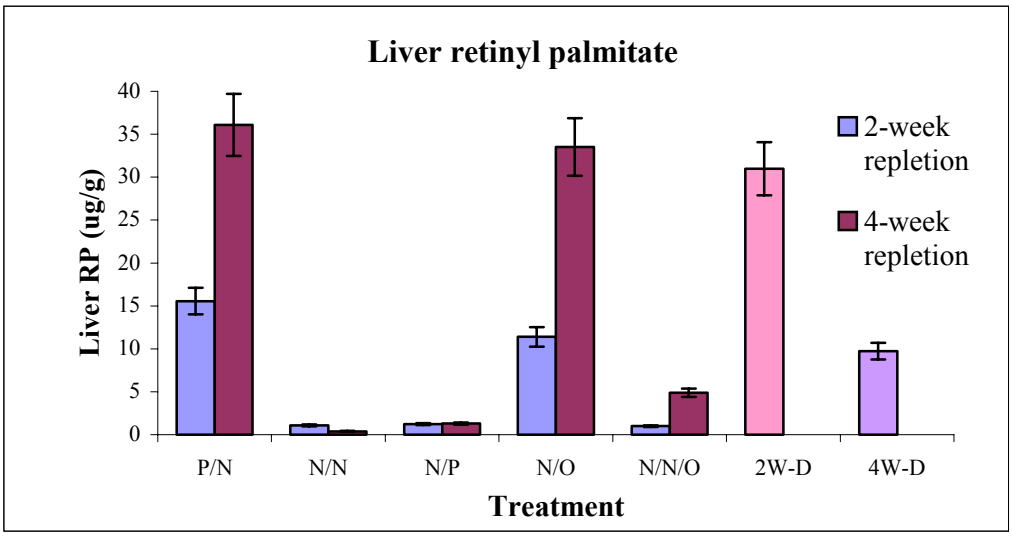

Figure 5.16 Comparison of liver retinyl palmitate concentrations

2W-D: 2-week depletion group; 4W-D: 4-week depletion group

Chapter 6 Summary and Conclusion

One of the major sources of dietary vitamins A and D is dairy products fortified with retinyl palmitate and vitamin D_3. As more and more people in this country consume reduced-fat dairy products, under-fortification of vitamins in such products rendered potential vitamin deficiency a public health concern (Murphy, 2001). Vitamin A was reported to be more stable in a skim milk system when water-based carriers (such as protein) were used (Fellman, 1991). This research had studied the potential of β-lactoglobulin (BLG) as a soluble and stable carrier for the vitamins A and D in skim milk system through shelf life and bioavailability studies.

To develop the protein-based fortifiers (Chapter 3), BLG-vitamin complexes were prepared in a molar ratio of 1:1 based on the binding characteristics of BLG with vitamins A and D (Wu, 1999; Wang, 1997) and dried by spray-drying and freeze-drying. Binding of vitamins with the protein was confirmed by fluorescence spectroscopy. High outlet temperature in the spray drying apparatus caused poor recovery and isomerization of vitamins compared with freeze-drying, though the latter is much more energy costly and applicable to relatively smaller scale of samples. Spray drying has been widely used for drying heat-sensitive foods, pharmaceuticals, and micro-encapsulation. Oxidation of anhydrous milk fat was significantly limited by micro-encapsulation in whey protein isolate by spray drying (Moreau, 1996). Future research should be conducted to optimize the drying protocol and determine whether spray drying still can be used to prepare BLG-vitamin complexes on a large scale.

Shelf life stability of BLG-vitamin complexes in HTST or UHT-processed skim milk stored in plastic or glass bottles was compared with regular oil-based fortifiers (Chapter 4).

Retinyl palmitate and vitamin D_3 were assayed using normal-phase and reversed-phase HPLC, respectively. No appreciable change of vitamin D_3 concentration was observed in HTST skim milk with various type of fortification, indicating vitamin D is more stable than vitamin A in fortified skim milk as shown in other studies (Indyk, 1996). Package materials did not affect shelf life of either vitamin when the homogenized samples were stored in a refrigerator. BLG-vitamin A was stable in HTST-processed skim milk during storage while Roche dry vitamin A (a starch-based emulsion) was not stable after 2 weeks of shelf life. Surprisingly, oil-based vitamin A fortifier was stable in fortified skim milk after thorough homogenization and HTST pasteurization in the NCSU dairy plant, which disagreed with other studies reporting sorption of vitamins onto the polyethylene material of plastic or paperboard containers (Smith, 1988; Paredes, 1996). Efficient homogenization may play an important role in preventing vitamin loss from degradation, separation, or adherence to the container. In UHT-processed skim milk, BLG-vitamin A dropped to 1/3 of the targeted fortification level right after UHT processing, possibly due to partial denaturing of the protein and vitamin A deposition to the sterilization system. In this trial, BLG-vitamin D exhibited good stability while oil-based vitamin D dropped to half the original level at the end of the 6-week shelf life. The slow decrease of both vitamins in UHT processed skim milk might be caused by oxidative degradation of vitamins by residual hydrogen peroxide from the sterilization procedure or sorption of vitamins onto the polyethylene material inside the paperboard cartons. Additional research is needed to fully define the causes for vitamin A and D loss from skim milk during shelf life studies.

Bioavailability of BLG-vitamin complexes was investigated using the vitamin-depleted rat model system (Chapter 5). Results from a serum 25-OH D RIA assay were used as an

indicator for vitamin D bioavailability. The group fed BLG-vitamin D-fortified skim milk had the highest level of serum 25-OH D among all groups, although their total vitamin D intake was higher than the other groups due to the better shelf life and stability of the vitamin D in the milk they were fed. With over-adequate intake of calcium, all treatment groups, including the negative control group that was vitamin D deficient throughout the study, had normal serum calcium, bone ash, bone calcium, and bone strength, probably due to the high calcium intake from skim milk. Other studies also reported or slightly sub-normal bones in vitamin D-deficient rats when the mineral supply is adequate (Toromanoff, 1997 and Kollenkirchen, 1991). It is likely that vitamin D is not needed for bone calcification but is responsible for maintaining extracellular calcium and phosphorus concentrations in a supersaturated state when dietary calcium is low, which results in mineralization of bone.

For vitamin A bioavailability, liver and serum vitamin A were used to indicate vitamin A status in the rat. After the 4-week depletion, liver vitamin A storage dropped dramatically while serum retinol concentrations were not significantly different from the baseline, similar to other studies (Gardner, 1993; Lewis, 1990). After the 4-week repletion with vitamin A, oil-based retinyl palmitate in skim milk repleted liver and serum vitamin A equivalent to vitamin A-containing AIN-93G pelleted rodent diet, indicating similar bioavailability of retinyl palmitate in skim milk and pelleted diet given the similar vitamin A intake in these two groups. The vitamin A content of the UHT-processed skim milk fortified with BLG-vitamin A complex was unexpectedly low, so a treatment with a similar vitamin A intake from skim milk fortified with the oil-based fortifier was designed to provide the equivalent amount of retinyl palmitate; the vitamin A intake between these two groups was similar.

Based on data from liver and serum vitamin A assay, there was no significant difference in bioavailability of retinyl palmitate between PFM and OFM.

In conclusion, BLG-complexed retinyl palmitate is not an appropriate fortifier for UHT-pasteurized skim milk due to apparently lower temperature stability. However, this complex appears to be stable in HTST-pasteurized skim milk; BLG-complexed vitamin D_3 is a more stable fortifier than the regular oil-based fortifier under the conditions tested in our shelf life studies. There was no difference detected between the BLG complexes and the oil-based fortifiers in bioavailability of vitamins.

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