

## ABSTRACT

LOVETT, MALLORYE DELORIS. Calcium Chloride and Vitamin D Fortified Beverages: Bioavailability in Wistar Rats. (Under the direction of J.C. Allen.)

Calcium and vitamin D deficiency is now recognized as an epidemic in the United States. Calcium and vitamin D play a critical role in the prevention of metabolic diseases including osteoporosis, osteomalacia, and rickets. Epidemiological research indicates that average intake of these nutrients is well below the RDA. Dairy products continue to be the main source of calcium intake. The major source of vitamin D is from sensible sun exposure. Greater intake of calcium and vitamin D has been correlated to a reduction in fractures, prevention of osteoporosis, and increased bone mass.

The objective of this study was to test effects of a water-soluble form of vitamin D and calcium chloride as fortifiers for an aqueous sports drink solution with a rat bioavailability assay.

A water-soluble vitamin D fortifying ingredient was prepared as a spray-dried complex with bovine beta-lactoglobulin. Vitamin D content of the complex was assessed by HPLC. Flavored beverages were formulated with various ratios of calcium and vitamin D in a 4x4 factorial design. Female Wistar rats were housed under incandescent lighting and randomly divided into the treatment and control groups. After a 4-week depletion phase, rats were given specialized drink formulations and low calcium, vitamin D-deficient diet for an additional six weeks. Blood and femur bones were removed for further analysis. Serum vitamin D was measured by ELISA.

Results demonstrate that fortified drink solutions could be accurately formulated to contain calcium chloride at 0, 1, 2 and 2.5 g Ca/L with palatability to rats. The vitamin D

content of the drinks was formulated to be 0, 10, 20, and 40  $\mu\text{g/L}$ . Serum vitamin D was significantly greater ( $p < .0001$ ) in rats receiving the vitamin D-fortified drinks.

Water-soluble vitamin-D can be used to fortify aqueous products with this fat-soluble vitamin to help facilitate the uptake of calcium. Regular consumption of flavored sports drink fortified with calcium and vitamin D may significantly increase dietary calcium and vitamin D intake.

Calcium Chloride and Vitamin D Fortified Beverages: Bioavailability in Wistar Rats

by

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## DEDICATION

This thesis is dedicated to my parents, sister, family and close network of friends. I would like to extend a very special thank you to my parents for providing a nurturing environment during this academic process. In addition, thank you for the myriad of ways in which you have supported and loved me in my determination to find and realize my potential and purpose in life.

To my sister, family and close friends who have played such important roles throughout my academic journey, I thank you. These networks of individuals have allowed me to continuously learn, grow, and enjoy the simple aspects of life. They have been a source of encouragement and inspiration to me throughout my life.

## BIOGRAPHY

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CHAPTER 1

**Literature Review**

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## **1.1 Preface**

The synergistic effects of calcium and vitamin D have been correlated with the prevention of certain chronic diseases including, osteoporosis, osteomalacia and rickets. Moreover, adequate amounts of these nutrients have been shown to play a significant role in the reduction and prevention of increased bone loss and susceptibility to falls and fractures.

Calcium has been shown as an essential nutrient that is responsible for proper bone and teeth mineralization. Calcium intake in the diet has declined in recent years, due to the avoidance of high calcium foods and/or the increased consumption of low-fat products. Milk is the primary source of calcium in the diet, but steady decline in milk consumption in recent decades has posed a significant issue.

Vitamin D has also been found to have a positive effect on bone development. One of the main food sources of vitamin D is milk. In addition, vitamin D can also be obtained from sunlight. Exposure to at least 15-20 minutes of sunlight can supply the daily requirement for this vitamin. However, vitamin D deficiency is still an issue in the United States because many individuals are not exposed to sunlight due to more indoor activities that limit exposure to the sun or in infants and elderly individuals who are home bound or institutionalized.

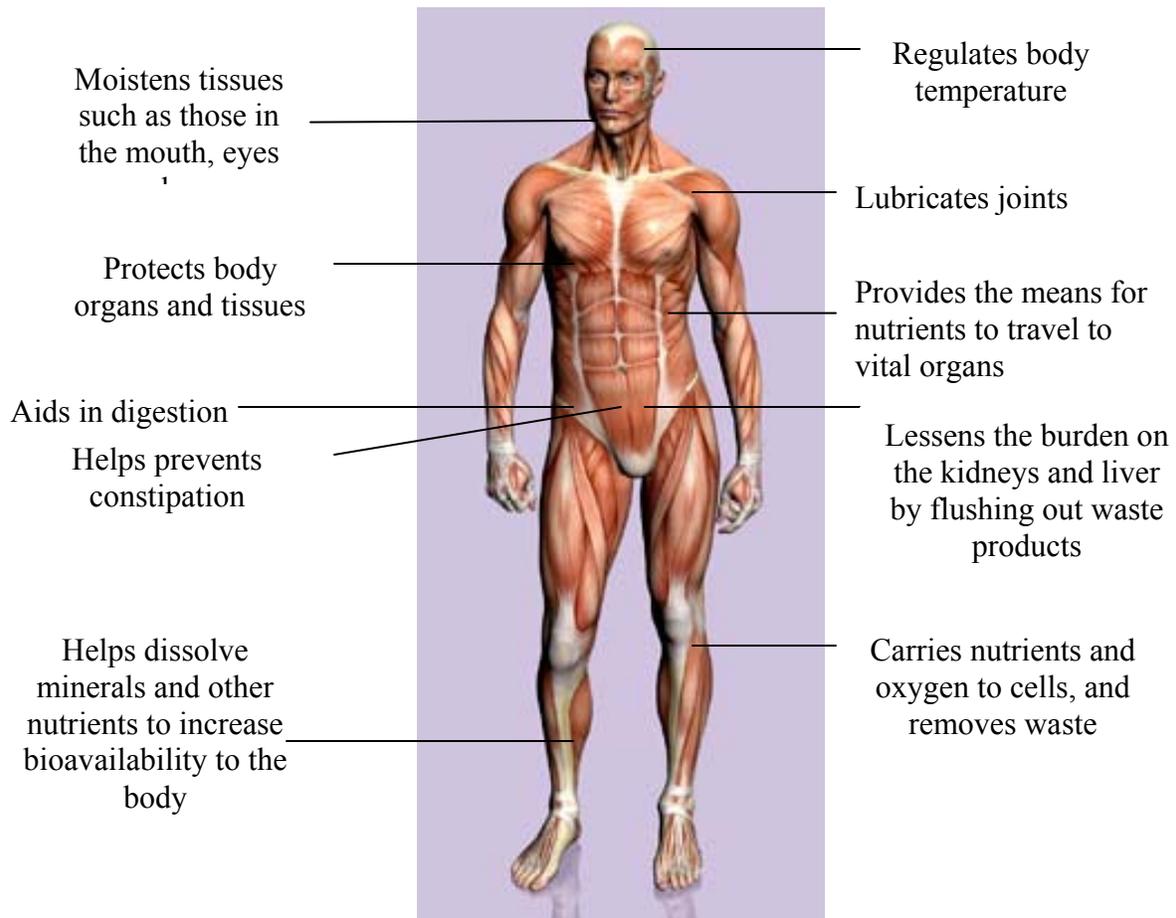
The bottled water market continues to grow at a faster rate than other beverage sectors. Consumers are aware of the health benefits associated with daily consumption of fluid water. Fortification of bottled water with nutrients, non-caloric sweetener, and flavor may increase the bottled water market exponentially.

In conclusion, the increased consumption of water is beneficial to human health. The fortification of water with nutrients and flavor has the ability to increase consumption globally while providing nutrients required for proper growth and development.

## **1.2 Introduction**

### **1.2.1 Water**

The most abundant constituent in the human body is water. The body is comprised of an estimated 60 to 70 percent water. Water provides fundamental nutrients necessary to sustain cellular homeostasis and life (Figure 1.1). In addition, water is the medium for various enzymatic and chemical reactions that occur in the body. Water facilitates the movement of nutrients, hormones, antibodies, and oxygen throughout the blood stream and lymphatic system (WHO, 2006). Water utilized by the body is obtained from various sources including drinking water, water in beverages and water obtained from food. Previous research studies have found an association between low intake of total water and some chronic diseases including breast cancer, and prostate cancer, and cardiovascular health (IOM, 2004). Even though, the essential benefits of water have been clearly defined, the current United States Department of Agriculture (USDA) MyPyramid does not include water as an important component (WHO, 2006). Moreover, previous research studies have not provided enough substantial evidence to establish Adequate Intake (AI) recommendations for water (IOM, 2004).



**Figure 1.1 Functions of Water in the Human Body**

The majority of all the major systems in the human body depend on water to facilitate normal body function (modified Mayo Foundation for Medical Education and Research, 2007).

### **1.2.2 Hydration Status**

Water homeostasis is maintained within narrow limits in the human body. Water balance is controlled through water loss from the urinary system, the skin, the respiratory system and the gastrointestinal tract. Water input from drinking water, water in beverages and water from food restores the fluids lost through the various excretory methods (D'Anci et al., 2006). Water imbalance affects the hydration status of the body. Dehydration is an imbalance in the water intake and output that has been associated with certain chronic diseases. Dehydration may adversely contribute to various chronic diseases, one in particular is osteoporosis (IOM, 2004).

### **1.2.3 Dehydration and Osteoporosis**

Osteoporosis is defined as a condition characterized by a decrease in bone mass and density that leads to increased bone fragility (Ringe and Schacht, 2004). Currently, there are no longitudinal research studies that have addressed the effect of dehydration on osteoporosis. However, short term research studies have been conducted on the role of dehydration and the alteration in bone mineralization. The findings suggest hydration status impacts body composition and bone mineral density in healthy individuals by effecting bone mineralization (IOM, 2004).

### **1.2.4 Bottled Water Consumption**

In 2003, bottled water emerged as the second largest commercial beverage category by volume in the United States. In subsequent years, the consumption of bottled water

continues to increase. This sector of the market is growing even more forcefully on a global scale but in the US, volume increase is unmatched (Decker, 2006).

In 2005, total US sales of bottled water were estimated between \$50 and \$100 billion annually and increasing approximately 7 to 10 percent annually (Gleick, 2004). Moreover, volume exceeded 7.5 billion gallons, a 10.7% advance over the previous year. This increase suggests that the average American consumes an average of 26.1 gallons annually. The average consumption of bottled water is more than any other beverage, other than carbonated soft drinks (CSDs). Even though CSDs continues to be the most consumed beverage by volume, the soft drink market has been struggling recently due to competition from the bottled water sector (Decker, 2006). As bottled water becomes more of a commodity purchase, industries are trying to expand the bottled water market by adding vitamins, minerals, sweeteners, flavor, etc. These line extensions have found niche markets that have increased sales in this beverage segment. Consumers' perceptions of these new products are that they are a healthier replacement to traditional CSDs (Decker, 2003).

### **1.2.5 Flavored Fortified (Functional) Water Drinks**

Flavored water has a stronghold in the beverage industry. Moreover, the fortified water beverage category is leading the industry. Consumers have a growing desire to find a beverage that not only refreshes and has an acceptable taste but also offers a functional benefit. Functional waters that have been fortified with vitamins and minerals are becoming increasingly acceptable among consumers. Functional beverages are projected to increase from \$10 billion in 2004, to a \$12.8 billion industry by 2009 (Decker, 2006).

### **1.2.6 Benefits**

Popkin et al. (2006) recently proposed guidelines for beverage consumption after completing an in-depth review of research investigating effects of fortified beverages on health. The article stressed the importance of a healthy diet including adequate intake of water for metabolism and normal physiological function (Popkin et al., 2006). The primary benefits of consuming functional water beverages are increased palatability, better hydration, physiological maintenance, and access to hydration. Continuous development of functional aqueous beverages may not increase the beverage market but may play an important role in nutritional health (Decker, 2003).

## **1.3 History of Fortification**

### **1.3.1 History**

Fortification is defined as the addition of nutrients to food constituents to maintain or improve the overall nutritional quality of the food (Yetley, 2004). In 1924, voluntary fortification of salt with iodine was a result of the increased prevalence of goiter among the US population. Iodization of salt led to a decrease in prevalence of goiter from 38.6% to 9%. Continued use of iodine-fortified salt nearly led to the elimination of goiter in the 1930s (Backstrand, 2002). Moreover, in 1933 vitamin D was adopted as a fortifier for fluid milk. Milk was fortified to combat the increasing prevalence of rickets, a deficiency of vitamin D that may cause abnormal bone formation (IOM, 2003).

The United States continued to address the role of fortification as systematic approach to alleviate nutritional deficiencies. In addition, throughout World War II the enlistees frequently had poor nutritional status. To combat the issues of poor nutritional status and

nutritional deficiencies, President Roosevelt assembled the National Nutrition Conference for Defense in 1941 which is currently referred to as the Food and Nutrition Board (FNB). A major result of this meeting was the recommendation for enrichment of flour and bread. Moreover, this was the first presentation of the Recommended Daily Allowances (RDAs). RDAs were established for various vitamins and minerals and some of these components were approved as fortifiers. Thiamin, niacin, riboflavin, calcium, vitamin D and vitamin A were approved as fortifiers for such food products as flour, bread, milk and margarine. To be utilized as a fortifier a given nutrient must reflect standards established by the FNB and the Council on Foods and Nutrition of the American Medical Association (AMA):

- *The intake of the nutrient, in the absence of fortification, is below the desirable level in diets of a significant number of people.*
- *The food from which the nutrient is to be derived is likely to be consumed in quantities that will make a significant contribution to the diet of the population in need.*
- *The addition of the nutrient is unlikely to create an imbalance of essential nutrients.*
- *The nutrient added is stable under proper conditions of storage and use.*
- *The nutrient is physiologically available from the food to which it will be added.*
- *There is a reasonable assurance against intake sufficiently in excess to be toxic (IOM, 2003).*

## 1.4 Calcium

### 1.4.1 Calcium source and Metabolism

Calcium is a major constituent of mammalian mineralization. Calcium aids in the normal growth and development of bone (Bronner and Pansu, 1999). The majority of calcium (~99%), an essential nutrient, is found in the bones and teeth. The remaining 1% is contained in the serum, extravascular fluid, muscle, and other tissues (Committee of Nutrition, 1999). This nutrient is regulated by several hormones including the Parathyroid Hormone (PTH), calcitonin, and vitamin D. These regulators, when operating appropriately, maintain calcium balance within narrow limits. A low intake of calcium stimulates the breakdown of bone to supply the needed calcium for normal physiological functions. Continued release of calcium from the bone causes a reduction in bone mass and increases the risk of osteoporosis. Good sources of calcium are abundant throughout the U.S. Calcium can be obtained from calcium-rich foods such as milk, dairy products and other fortified foods such as cereal and juice beverages (IOM, 1997). The Institute of Medicine (IOM) recommended adequate intake of calcium ranges from 1200- 1300 mg/day (IOM, 1997).

**Table 1.1 Recommended Adequate Intake by the IOM for Calcium**

<b>Male and Female Age</b>	<b>Calcium (mg/day)</b>	<b>Pregnancy &amp; Lactation</b>
0 to 6 months	210	N/A
7 to 12 months	270	N/A
1 to 3 years	500	N/A
4 to 8 years	800	N/A
9 to 13 years	1300	N/A
14 to 18 years	1300	1300
19 to 50 years	1000	1000
51+ years	1200	N/A

Source: Institute of Medicine

### **1.4.2 Bioavailability and Digestion**

The bioavailability of a nutrient encompasses the absorption, transport, and utilization of the nutrient in the body. Fortification of food products has led to increased concern among the nutritional community. Nutritionists are concerned that the bioavailability from fortified sources is not nutritionally equivalent to naturally occurring calcium-rich foods as it relates to absorption (Bronner and Pansu, 1999). Absorbability of calcium is directly related to calcium bioavailability (Kruger et al., 2003). Calcium bioavailability and absorption can be enhanced or inhibited by several nutritional factors. Bronner et al. (1999) have identified fat intake and degree of calcium soaps as factors that may inhibit the absorbability of calcium. Moreover, calcium absorption is inhibited by food sources containing significant amounts of oxalic acid (spinach, sweet potatoes, rhubarb, and beans) and phytic acid (seeds, nuts, grains and soy isolates) (IOM, 1997).

Calcium is transported by active transport and passive diffusion. Active transport is defined as the portion of calcium entry, intracellular diffusion, and extrusion which requires metabolic energy. Active transport is the primary mechanism of calcium absorption at low and moderate intake and increases in the presence of 1,25 dihydroxyvitamin D, the active form of vitamin D. Conversely, passive diffusion is imperative with high intakes of calcium diffusion across the intestinal mucosa (Bronner et al., 1999 and IOM, 1997). Calcium is absorbed in the small intestine (duodenum) with favorable low pH (<6) (IOM, 1997).

### **1.4.3 Functions**

The relationship between inadequate intake of calcium and chronic disease has been extensively studied (Nordin, 1997).

### 1.4.3a – Osteoporosis and Calcium

The prevalence of osteoporosis increases with age. Osteoporosis is a multifactorial disease in which nutrition plays a vital role. The risk factors of osteoporosis are classified as modifiable, unmodifiable, and treatable (Figure 1.2). Osteoporosis is characterized by continued calcium deficiency which leads to negative calcium balance. In this physical state the body relies on calcium from breakdown of the skeletal bone, which provides a reserve supply of calcium in a state of calcium deficiency (IOM, 1997 and Nordin, 1997). Osteoporosis increases bone fragility and susceptibility to increased risk of fractures (IOM, 1997). Nonetheless, several research studies have shown that increasing dietary calcium via fortification and/or other supplementary vehicles may supply enough calcium to meet the body's metabolic needs (Nordin, 1996 and Ringe and Schacht 2004).

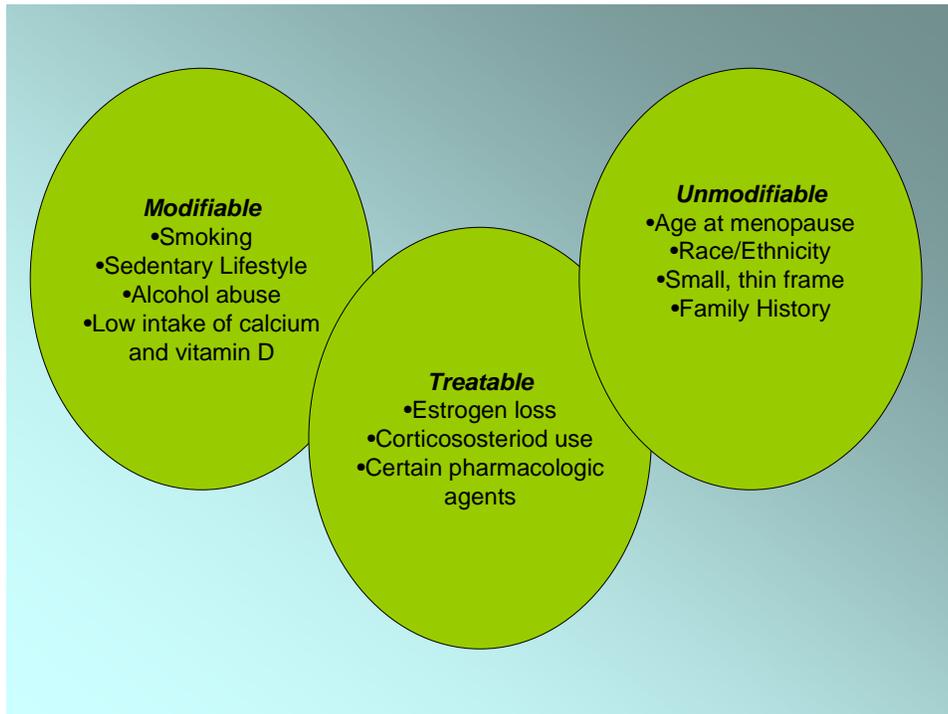


Figure 1.2- Osteoporosis Risk Factors

### **1.4.3b- Hypertension and Calcium**

Calcium may have a plausible role in the lowering the risk of hypertension. Several studies have investigated the role of increased supplementary calcium and/or low fat dairy (good sources of calcium) products and found a measurable decrease in systolic blood pressure among normotensive and hypertensive adults (IOM, 1997).

### **1.4.3c- Cancer and Calcium**

Calcium plays a pivotal role in intracellular signaling and controls many different cell processes. Calcium has been shown to play a role in cell growth. Furthermore, calcium participates in selective induction of programmed cell death or apoptosis of cancer cells (Lee et al., 2006 and Sergeev, 2005).

### **1.4.4 – Calcium Fortification**

Previous research has reported the overall benefit of adequate calcium intake and positive correlation between bone health and reduction of various types of diseases. The awareness of the beneficial effects of calcium has led to an increase in fortified food products. Currently, several products are fortified with calcium, including breakfast cereals, orange juice, milk, yogurt, and cheese (Bowen, 2004 and IOM, 1997). Table 1.2, illustrates the United States Recommended Dietary Allowances for calcium and optimal calcium intake.

**Table 1.2 RDA Calcium**

Group	RDA 1989 <sup>a</sup> (mg/day)	Optimal daily intake <sup>b</sup> (mg/day)	Adequate Intake 1997 <sup>c</sup>
<i>Infants</i>			
Birth – 6 months	400	400	210
6mths – 1yr	600	600	270
<i>Children</i>			
1 – 5 yrs	800	800	
1-3 yr			500
6-10 yrs	800	800-1200	
4-8 yr			800
<i>Adolescents/Young Adults</i>			
11-24yrs	1200	1200-1500	
9-18 yr			1300
<i>Men</i>			
25-65yrs	800	1000	
Over 65yrs	800	1500	
<i>Women</i>			
25-50yrs	800	1000	
<i>Men and women</i>			
19-50 yr			1000
Over 50			1200
<i>Over 50yrs (postmenopausal)</i>			
On estrogens	800	1000	
Not on estrogens	800	1500	
Over 65yrs	800	1500	
Pregnant or lactating	1200	1200-1500	
≤18 yr			1300
19-50			1000

<sup>a</sup> National Research Council. Recommended dietary allowances. 10<sup>th</sup> ed.

Washington DC: National Academy Press, 1989:174-184

<sup>b</sup> NIH Consensus Statement. Optimal calcium intake. JAMA 1994; 272: 1942-1948

<sup>c</sup> Food and Nutrition Board, Institute of Medicine (1997) Dietary Reference Intakes for Calcium, Phosphorus, Magnesium, Vitamin D and Fluoride. National Academy Press, www.nap.edu

## **1.5 – Calcium Chloride**

Calcium chloride is a chemical composed of calcium and chloride. This chemical is highly soluble in water. Calcium chloride is a commonly utilized industrial agent in products such as pickles to give a salty taste while not increasing the amount of sodium. It is added as an ingredient in canned vegetable to maintain freshness, as a brine for fresh cut fruits and vegetables to prevent color changes and increase shelf stability, and in some sports drinks as an electrolyte. Even though calcium chloride has the above beneficial effects it is not an ideal fortifier because of the bitterness it contributes to the food product (Luna-Guzman and Barrett, 2000 and Varela et al. 2007). Nonetheless previous research studies have indicated if calcium chloride is used in combination with sucrose it will suppress the undesirable bitterness attributed by calcium chloride (Lawless et al., 2003).

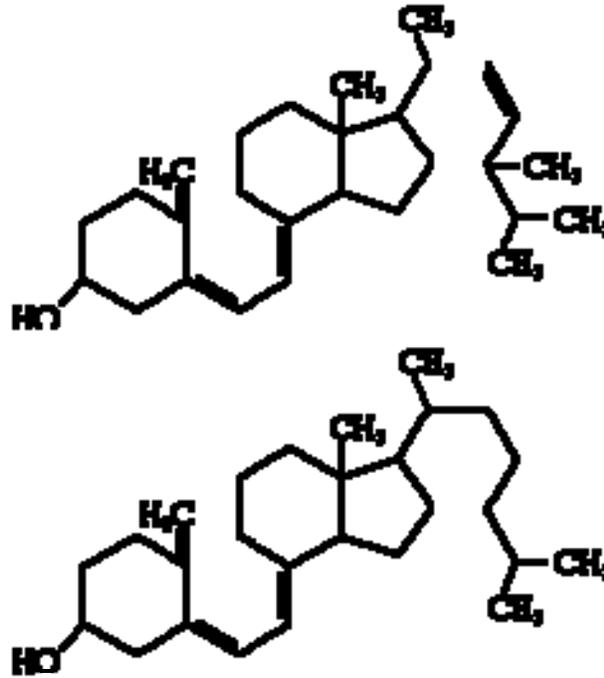
## **1.6 – Vitamin D**

### **1.6.1 – Source, Structure, and Synthesis**

Previously little was known about the evolutionary perspective of vitamin D (calciferol). In 1919, a scientist named Edward Mellanby discovered the importance of vitamin D on development, growth and maintenance of a healthy skeletal system when investigating the prevalence of rickets (Holick, 2003). In addition, throughout the years of 1924 - 1927 researchers investigated vitamin D found in oily fish and in fish liver oils. It was believed that the vitamin D in these components was due to dietary intake of vitamin D from phytoplankton and zooplankton. These scientists were able to demonstrate that seasonal variation in vitamin D could be noticed in oily fish and fish liver, with the highest

amounts of vitamin D during the summer months and a lower content of vitamin D during the winter months (Holick, 2003 and 2005).

As illustrated in Figure 1.3, vitamin D comes in various forms, but the two most prominent forms are ergocalciferol (vitamin D<sub>2</sub>) and cholecalciferol (vitamin D<sub>3</sub>) (IOM, 1997). Vitamin D<sub>2</sub> is derived from yeast and plant sterol, and is the usual form of vitamin D employed during fortification. On the other hand, vitamin D<sub>3</sub> is obtained when radiant energy (UV light) from the sun interacts with 7-dehydrocholesterol, a precursor of cholesterol, found in the skin. Due to the fact that the body is capable of producing cholecalciferol, vitamin D is not defined as the classical vitamin. A more accurate description of vitamin D is a prohormone; which indicates that the vitamin is metabolized to a biologically active form that functions as a steroid hormone. Nonetheless, since the importance of vitamin D has been discovered it has been classified among the lipid-soluble vitamins (IOM, 1997 and Lipkin and Lampricht, 2006).



**Figure 1.3 Structures of Vitamin D<sub>2</sub> and D<sub>3</sub>**

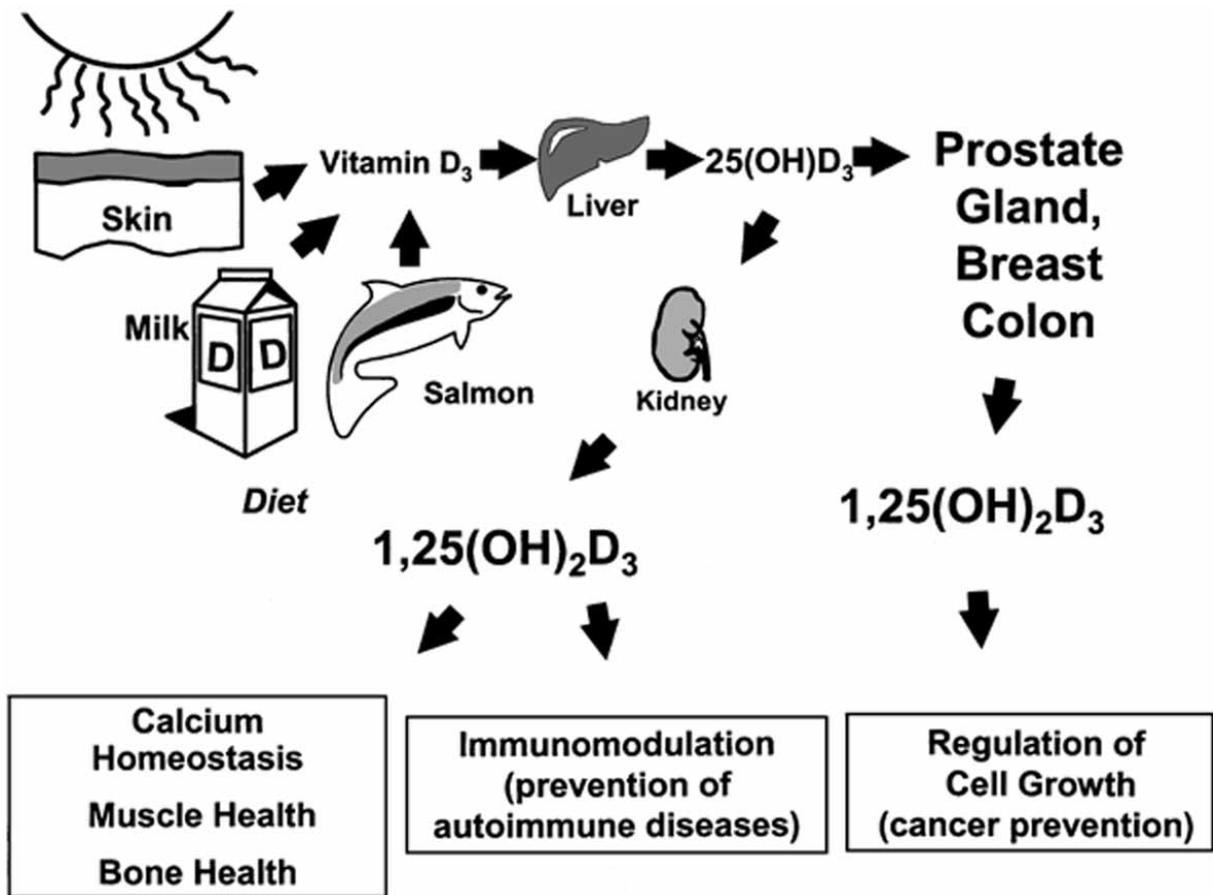
Vitamin D<sub>2</sub> top, Vitamin D<sub>3</sub> bottom (IOM, 1997)

The primary sources of vitamin D in nature are very few foods including fish liver oils, the flesh of fatty fish, and eggs from hens fed vitamin D enriched diet. Fortified food products also provide vitamin D including milk, margarine, breads and fortified breakfast cereals. Throughout the world the major source of vitamin D is via exposure to sunlight (Hollis, 2005 and IOM, 1997).

### **1.6.2- Absorption and Digestion**

Enzymes in the liver and kidney convert the prohormone form of vitamin D to the active form of vitamin D (Figure 1.4). When adequate amounts of vitamin D are ingested via food, vitamin D is absorbed along with the dietary fat in the intestine. Exposure to sunlight

converts 7-dehydro-cholesterol to vitamin D. Vitamin D binds to the protein carrier in the blood and is transported to the liver. While in the liver, vitamin D is hydroxylated to 25-OH vitamin D, the circulating form, and further metabolized to the active hormone form, 1, 25 dihydroxy vitamin D by the kidney. The active vitamin D is then transported to the target tissue in the body. Moreover, the metabolism of vitamin D is feasible due to a specific protein carrier. Vitamin D that is circulated throughout the body is bound to a specific protein carrier, vitamin D binding protein (DBP) (Rowling et al., 2006). DBP binds the metabolites of vitamin D according to affinity, therefore DBP binds accordingly  $25(0H)D > 24,25(OH)_2D > 1, 25(OH)_2D > \text{vitamin D}$ . This binding capacity facilitates the delivery of vitamin D to various sites for metabolism, storage and action (Dusso et al., 2005). Vitamin D is stored in the liver (Holick, 2003).



**Figure 1.4- Production, Metabolism and Biological Functions of Vitamin D<sub>3</sub>**

(Holick, 2003)

### 1.6.3 – Function

After vitamin D has been metabolized into its active form it can contribute to the biological activity of the body. Vitamin D<sub>3</sub> plays a significant role in the regulation of blood calcium by increasing absorption via the small intestine, reducing calcium excretion via the kidney and regulating calcium loss from the bones. The parathyroid hormone regulates the production of 1,25-(OH)<sub>2</sub>D<sub>2</sub> when calcium blood levels are low (IOM, 1997).

Moreover, vitamin D plays a significant role in the maintenance of serum calcium homeostasis and phosphorus homeostasis within the range that supports normal neuromuscular function, bone calcification and other cellular processes. For example, calcium is a critical component of muscle contraction, nerve pulse transmission, blood clotting, and membrane structure. Additionally, calcium and phosphorus work synergistically to promote normal bone mineralization. Maintenance of calcium and phosphorus within narrow limits by vitamin D is very important for all living organisms (IOM, 1997).

#### **1.6.4 – Effect of Vitamin D Deficiency**

Vitamin D deficiency is of global concern. Everyone is at risk of vitamin D deficiency due to many confounding factors (Holick, 2005 and Jasinghe et al., 2006). Inadequate vitamin D in the body elevates serum parathyroid hormone (PTH), which facilitates an increase in bone resorption. The condition of prolonged insufficient vitamin D is termed rickets in children and osteomalacia in the adult population. These conditions are common in children and women who have inadequate amount of vitamin D in the body. Rickets and osteomalacia may occur in breastfed infants who do not receive vitamin D supplementation and in both children and women who have inadequate vitamin D intake from food and/or lack of exposure to ultraviolet light for photosynthesis of vitamin D (Vieth, 2001).

#### **1.6.5 – Vitamin D Fortification**

In 1941, President Roosevelt issued a request for fortification of food products with vitamin D. His goal was to ensure that consumers had a sufficient amount of vitamin D in

the diet to alleviate the epidemic of vitamin D deficiency in industrialized communities. This outcry from President Roosevelt resulted in vitamin D-fortified milk. Currently, there are many other food items fortified with vitamin D including yogurts, cereals, orange juice, and nutritional bars. However, with additional fortification of certain food products with vitamin D, research studies have continuously reported that the current fortification is inadequate or not found in enough different food products to prevent vitamin D deficiency (IOM, 2003).

**Table 1.3 – 1997 RDI Adequate Intakes for Vitamin D**

<i>RDI for Vitamin D</i>	$\mu\text{g./day}$ ( $5\mu\text{g} = 200\text{IU}$ )
<b>Babies</b>	
<b>Birth-12months</b>	5
<b>Children</b>	
<b>1-13yrs</b>	5
<b>Adults</b>	
<b>14-50yrs</b>	5
<b>51 to 70yrs</b>	10
<b>Over 70yrs</b>	15
<b>Pregnant Woman</b>	5
<b>Nursing Women</b>	5

Source: Institute of Medicine

### 1.7 – Vitamin D Receptor (VDR)

Vitamin D nutrition affects health beyond bone density. Vitamin D is able to function in various capacities via a signaling mechanism with serum vitamin D and VDR, a soluble high-affinity receptor protein (Vieth, 2001). A widespread distribution exists for VDR in the body including the pancreas, lymph nodes, adrenal medulla, excreta; the vitamin D is dispersed into the cell membrane and transferred via the cytoplasm to the nucleus where the binding occurs (Holick, 2003).

VDR, a nuclear receptor for  $1\alpha,25(\text{OH})_2\text{D}_3$ , the active form of vitamin  $\text{D}_3$ , plays a critical role in bone formation. VDR is responsible for activation of calbindin, a calcium

transport protein in the small intestine. Moreover, the distribution of VDR throughout the body demonstrates the various functions  $1\alpha,25(\text{OH})_2\text{D}_3$  beyond mineral and skeletal homeostasis (Holick, 2003 and Veith 2001). Therefore, vitamin  $\text{D}_3$  is responsible for cell proliferation and inhibition or maturation of normal and tumors cells. Tanaka et al. investigated the effect of vitamin D on preleukemic cells in rats that were vitamin D sufficient. The researchers found that  $1\alpha,25(\text{OH})_2\text{D}_3$  and VDR work synergistically to inhibit growth and differentiation of cancerous cells (Holick, 2003).

### **1.8- Calcium and Vitamin D Interaction**

Adequate amounts of calcium and vitamin D throughout life helps reduce the risk of osteoporosis and reduced risk of fractures and falls (Calvo et al., 2005 and Iwamoto et al., 2004). The synergistic interactions between calcium and vitamin D have been well documented. Iwamoto et al. discovered that vitamin D supplementation had a positive effect on bone growth of young rats receiving a normal or low calcium diet. The supplementation stimulated intestinal calcium absorption and prevented bone resorption (Iwamoto et al., 2004).

### **1.9 – Calcium Chloride/Vitamin D Fortification**

Scientific research has continued to illustrate the importance of vitamins and minerals on human health. These findings have led to an increase in fortification of various food products. Moreover, United States federal government believes that fortification contributes to improving the nutritional status of the citizens (Backstrand, 2002 and IOM, 2004).

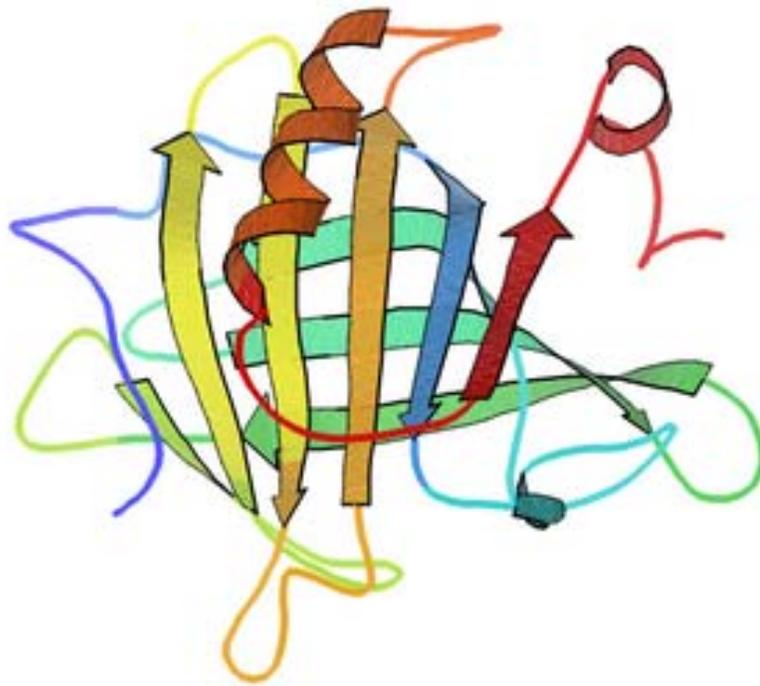
The Food and Drug Administration (FDA) has classified calcium chloride as a good source of calcium and a good quality fortifier (Luna-Guzman and Barrett, 2000 and Varela et

al. 2007). Therefore, many manufacturers utilize calcium chloride as a fortifier. However, manufacturers must be encouraged to utilize vitamin D, magnesium and riboflavin as fortification options to significantly improve intake of these substance. Even though vitamin D fortification is not as prevalent as calcium fortification, there is evidence that calcium and vitamin D work synergistically to significantly reduce the risk of chronic diseases, including osteoporosis and some forms of cancers (Calvo and Whiting, 2006).

### **1.10- Beta-lactoglobulin (BLG)**

BLG is a small protein that is a major component of whey protein. It is a polypeptide composed of 162 amino acids. It is naturally found in milk of various mammals including cows. BLG is soluble in dilute salt solution and is stable in a solution at the pH of the human stomach.

Research studies have investigated the binding ability of vitamin D to BLG, and found that vitamin D can bind to BLG. The binding of these two molecules will produce a complex that may be stable and soluble in aqueous solutions (Wang et al., 1997). This complex may be ideal for the fortification of an aqueous bottled beverage (Kontopodis et al., 2004).



**Figure 1.5- Structure of Beta-Lactoglobulin**

Source: [www.Bio-Rad.com](http://www.Bio-Rad.com)

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CHAPTER 2

**BLG-Vitamin D Binding By Spray-Drying**

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## 2.1- Introduction

Beta-lactoglobulin (BLG) has many functional properties including a transportation mechanism of molecules such as vitamins. BLG tightly binds to retinol (vitamin A), cholesterol and vitamin D (Kontopidis et al., 2004). Lipophilic vitamins structurally bind to the hydrophobic core of the BLG molecule. Previous studies suggest that BLG has a higher affinity for vitamin D compared to other lipophilic vitamins (Wang et al., 1997). Spray drying is an application that can facilitate the binding of beta-lactoglobulin and lipophilic vitamins.

The spray drying process is a commonly used method for dehydration of fluids. This process has been applicable to processing of milk, whey, buttermilk, butter and ice cream mixes. Food and pharmaceutical industries have investigated spray-drying as a means of incorporating vitamins and medication into various application systems (Ameri and Maa, 2006). However, with these applications the heat from the spray drying equipment is of concern. Heat can lead to denaturation of  $\beta$ -lactoglobulin and cause the complex to dissociate. In order to produce a BLG –vitamin complex, heat must be controlled to prevent denaturation.

The purpose of the experiment was to spray dry a complex of BLG and vitamin D that can be used as a fortifier of an aqueous solution. The gathered information was used to create a model Ca/vitamin D fortifier that could be used in an animal bioavailability experiment.

## **2.2 - Materials and Methods**

### **2.2.1- $\beta$ -lactoglobulin and Vitamin D Complex Preparation**

BioPure  $\beta$ -lactoglobulin was purchased from Davisco Foods International, Inc. (Le Sueur, MN). Vitamin D<sub>3</sub> (MW 384.65) and vitamin D<sub>2</sub> (MW 396.65) were purchased from Sigma Chemical Company (St. Louis, MO) and Roche Vitamins Inc. (Nutley, NJ), respectively. Four hundred ml of 2%w/v of  $\beta$ -lactoglobulin (~8g) in DI water was prepared. The solution was mixed on a magnetic stirring plate at low speed (2) to prevent foaming until a homogenous clear solution was obtained. This technique was employed to prevent the denaturation of BLG. When BLG is exposed to high speeds there is an increase in protein breakdown and also reduction in the binding capacity of BLG to vitamin D. Moreover, because of the sensitivity of vitamin D to light denaturation all work conducted with vitamin D was performed under dim yellow light and amber containers enclosed in aluminum foil to prevent degradation of vitamin D from direct light exposure.

### **2.2.2 – Spray Drying $\beta$ -lactoglobulin and Vitamin D Complex**

The complex was spray dried on a pilot scale dryer (Annhydro, Denmark). Prior to conducting the experiment, drying conditions were optimized to obtain parameters of 120°C inlet air temperature and 68-70°C outlet temperature. Earlier experiments have shown that these conditions would protect both BLG and vitamin D. The system was flushed with deionized water via a MasterFlex peristaltic pump (Model 7518-10, Cole-Parmer Instrument Co., Vernon Hills, IL) to stabilize the unit before the solution could be added. The protein-vitamin stock solution was pumped into the machine at a flow rate ~2 ml/min while

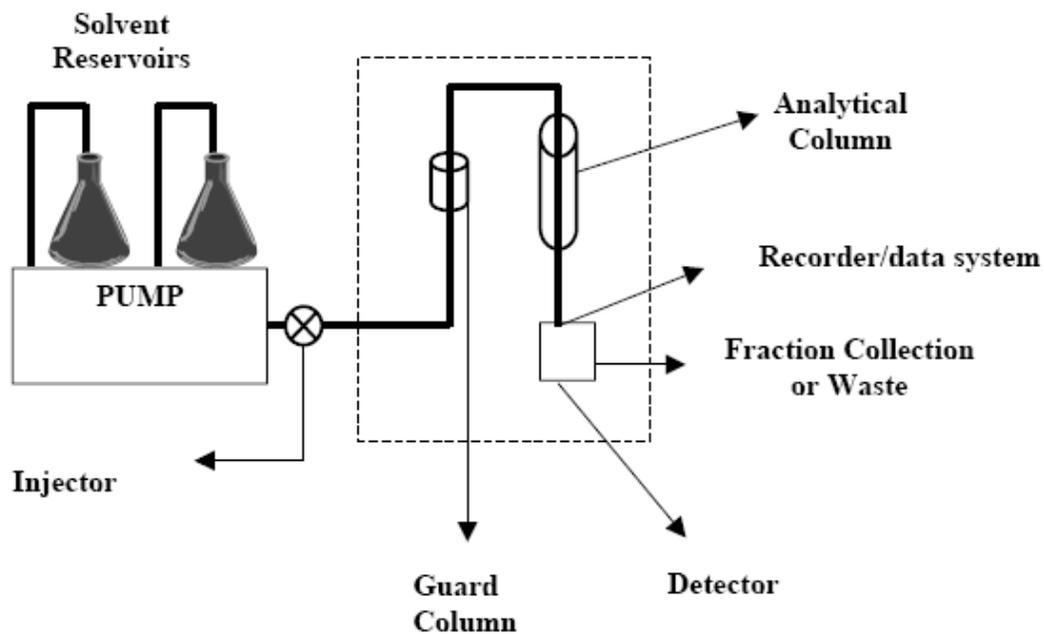
continuously monitoring the inlet and outlet temperatures to ensure that heat denaturation would not occur.

### **2.2.3 – Powder Recovery**

To ensure adequate recovery, the powder was weighed prior to flushing the system with deionized water. Simultaneously the system was turned off to cool and air flow was ceased to prevent loss of powder. Next, the powder was weighed, placed in an amber vial with aluminum foil surrounding the exterior, flushed with nitrogen, and stored in the freezer (-20°C) for further analyses.

### **2.2.4 – Vitamin D Analysis**

The vitamin D<sub>3</sub> content of the β-lactoglobulin-vitamin D complex was determined by High Performance Liquid Chromatography (HPLC) (Figure 2.1) using vitamin D<sub>2</sub> as internal standards as described by Liu (2003). HPLC analyses were performed on a Waters Millipore Automated Gradient Controller with UVIS linear detector and a manual loading injector (Waters Associates, Milford MA). Reversed-phase 4.6 x 250nm Vydac TP201 C18, 5 μ micron column with a guard column (Vydac, Hesperia, CA) was used with a mobile phase consisting of acetonitrile/ethyl acetate/chloroform (88:8:4; v/v/v) at a consistent flow rate of 1 ml/min. Methanol was used to wash and equilibrate the column before and after each sample injection. A wavelength of 264 nm was used to quantify the results and a Dynamax Software Package (Waters Associates) was used to integrate the peak areas of vitamin D. The following flow parameters were utilized during the analyses of the protein-vitamin complex based on an internal standard curve consistency of vitamin D<sub>2</sub> and D<sub>3</sub> (Table 2.1)



A systematic diagram of a HPLC (not drawn to scale). Columns and detector may be thermostated, as indicated by the dashed line, for operation at elevated temperature

**Figure 2.1- HPLC Analysis of Vitamin D in BLG- Vitamin D Complex**

(Eledah, 2005)

**Table 2.1- HPLC Program Flow**

Time	Flow	%A*	%B*
0.0	1.0	100	0
22.0	1.0	100	0
23.0	2.5	0	100
32.0	2.5	0	100
33.0	2.5	100	0
37.0	2.5	100	0
38.5	1.0	100	0

\*A is acetonitrile: ethyl acetate: chloroform (88:8:4 v/v/v) and B is Methanol.

### 2.2.5 – Standard Curve and Recovery of Vitamin D from Complex

Vitamin D<sub>2</sub> and D<sub>3</sub> stock solutions were made by adding 0.02 g of vitamin powder to 100 ml of methanol for a final concentration of 200 ug/ml. The concentrations of vitamin D<sub>2</sub> and D<sub>3</sub> were determined by a 1:100 dilution by obtaining the absorbency of the solution at 264 nm on a Genesys 2 spectrophotometer (Spectronic, Thermo Electron Scientific Instruments Co. Madison WI). The concentration calculations were determined by the following formula:

$$C = A/E^\circ \text{ or } A = E^\circ BC$$

C= absorption concentration

A= absorbance @ 264nm

E° = extinction coefficient; vitamin D<sub>2</sub>= 460; vitamin D<sub>3</sub>= 485

B= length (1cm)

Standard solutions were prepared by adding predetermined amounts of vitamin D to 4 ml amber vials according to Table 2.2. The vials were filled with 0.5 ml of methanol to re-dissolve the vitamin D. Fifty µl of each standard solution was manually injected to the HPLC system with the use of a syringe (Hamilton Co. Reno, Nevada microliter #805) for analysis.

**Table 2.2 – Vitamin D<sub>3</sub> (Cholecalciferol) Standards**

<b>Vial #</b>	<b>Vitamin D3 working solution (2.5 µg/ml, 20µl = 0.05µg)</b>	<b>Vitamin D2 working solution (2.5 µg/ml, 60µl = 0.15µg)</b>
#1	20 µl	60µl
#2	40 µl	60µl
#3	80 µl	60µl
#4	120 µl	60µl
#5	160 µl	60µl

## **2.2.6 – Assay of Vitamin D**

### **2.2.6a- Saponification**

A 0.02 g portion of vitamin D<sub>3</sub>-fortified β-lactoglobulin powder was removed from the stock spray dried powder and placed in 45-ml screw-on top tube. Fifteen ml of DI water was added to the sample and spiked with 100 µl D<sub>2</sub> (internal sample). Fifteen ml of 1% ethanolic pyrogallol (made freshly daily) was added. The solution was cooled by flushing under nitrogen while adding ~6.0 g of KOH pellets. Immediately the tubes were placed in ice to facilitate dissolution of the KOH pellets and control temperature of the samples. The tubes were wrapped with aluminum foil and shaken in darkness at room temperature overnight.

### **2.2.6b- Extraction**

Following saponification, samples were transferred to 125-ml separatory funnels with 15 ml of DI water used to rinse the saponification tubes. An additional 5 ml of ethanol and 45 ml of hexanes were added to the separatory funnels, the content was shaken for 1 minute

and allowed to stand for 4 minutes. After the 4-minute time period, the aqueous layer (hexanes) was transferred to the Erlenmeyer flask and the organic layer was transferred to a 250-ml separatory funnel. The organic layer was washed two more times with 45 ml hexanes as described above. The pooled hexanes were placed in a 250-ml separatory funnel and 50 ml of 5% KOH was added. The mixture was shaken and allowed to stand for 4 minutes, and the aqueous solution was discarded. Fifty ml of DI water was added to the organic solution in a 250 ml separatory funnel, the mixture was shaken for one minute, allowed to stand for 4 minutes, and the aqueous solution was discarded. An additional 50 ml of 55% ethanol:water was added to the organic solution contained in the separatory funnel, the mixture was shaken and allowed to stand for 10 minutes. The organic layer was transferred to the previously-collected hexane portion in a washed round bottom flask. The contents of the round bottom flask were placed on a Rotarvapor containing a water bath (Rotarvapor, BÜCHI, Switzerland) to evaporate the hexanes at 40 to 50°C. After the round bottom flask was dry, 5 ml of hexanes was immediately added to the flask containing the vitamin D residue. The contents were transferred to test tubes. The round bottom flask was washed two more times with 3 ml hexanes, and contents were also collected and placed in test tubes. The solvent content of the centrifuge was removed under nitrogen flush. Once dried the residue was re-dissolved in 500 µl methanol. The methanol residue mixture was filtered through 0.45-µm syringe filter into a 2-ml amber vial, samples were flushed with nitrogen and kept at -20°C until needed for further analysis.

### **2.2.7 – Recovery of Vitamin D from BLG Powder**

Fifty  $\mu\text{l}$  of the extracted sample was injected into the HPLC system. Standards were injected before injecting the unknown samples. Area under the curve (AUC) was recorded for vitamin D<sub>2</sub> and D<sub>3</sub>, respectively, Figure 2.2. The retention time was also obtained and recorded for the peak values under both vitamins (Table 2.3).

### **2.3 – Results**

Figure 2.3, shows the vitamin D standard curve. In addition, after spray drying the vitamin D-protein complex was analyzed and quantified by HPLC analysis (Table 2.4). The quantity of vitamin D in the protein vitamin complex was calculated based on the standard curve. The proposed vitamin D content highly correlated with the actual vitamin D contained in the powder (approximately 98% accuracy). An average concentration of vitamin D in BLG complex was calculated and used to design a model system for the animal study (Chapter 3). Additionally, the vitamin D content of the diet was analyzed. The results suggested that vitamin D was not only present in the normal diet but was also present in the vitamin D deficient diet. To further investigate the presence of vitamin D open and unopened samples of the diets were tested. The calculated vitamin D content from the samples yielded higher amounts of vitamin D than the manufacture's specifications (Table 2.5). Therefore, these results may have impacted the outcome of the bioavailability assay (Chapter 3).

### **2.4 - Discussion**

$\beta$ -lactoglobulin has been shown to bind with vitamin D (Kontopidis, 2004 and Wang et al. 1997). This complex has been shown as an effective fortifier of aqueous solutions

(Eledah, 2005 and Reynolds, 2005). In addition, the data confirmed that proposed vitamin D content was achievable.

The extraction and HPLC methods by Murphy et al. (2001) have been proven as a successful method of determination of vitamin D in fluid milk. Even though these methods were applied to a fat-containing substance, these methods were applied to quantify vitamin D<sub>2</sub> and D<sub>3</sub> in an aqueous solution. The HPLC chromatograph has unexpected peaks, when compared to studies in which the matrix (a complex system, i.e. milk or food component) did not exist (Faulkner et al., 2000 and Murphy et al., 2001). These deviations may be due to the use of deionized water as the solvent or noise from the HPLC system.

These findings help facilitate the development of a model Ca/vitamin D fortifier for use in an aqueous solution. The fortifier complex was tested for bioavailability in a rat model system (Chapter 3).

## **2.5 – Conclusion**

Additional work should focus on a better analytical method designed to ensure reproducible recovery of vitamin D. Moreover, due to the fact that vitamin D is fat soluble, the majority of the vitamin D fortification research is conducted in fat containing beverages. There is limited published data concerning vitamin D fortification of aqueous solution. This research has shown that the vitamin D and BLG complex is a good vitamin D carrier to be utilized in aqueous solutions.

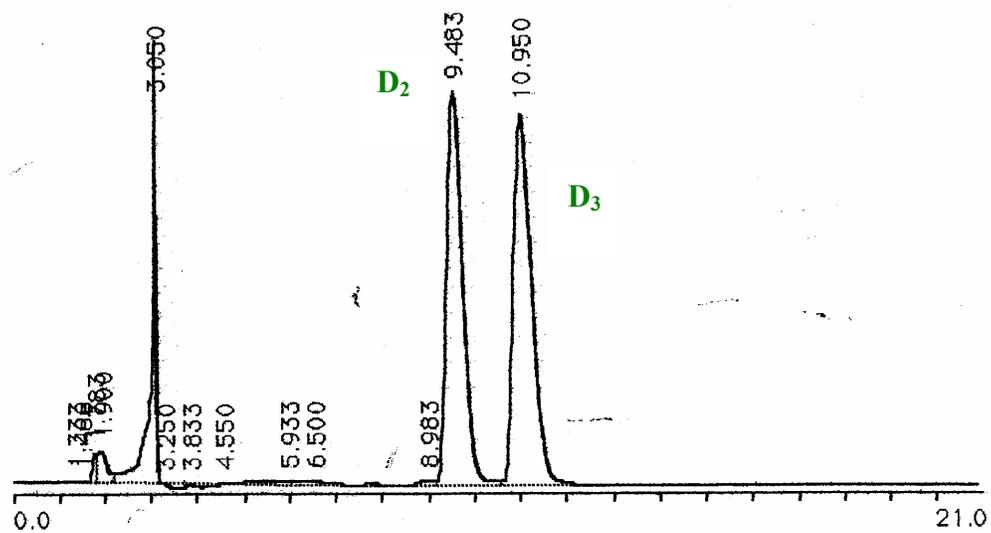


Figure 2.2 – HPLC Chromatogram of Vitamin D

**Table 2.3 – Retention Time and AUC for Vitamin D<sub>2</sub> and D<sub>3</sub>**

<b>Sample</b>	<b>Injection (<math>\mu</math>g)</b>	<b>Retention Time</b>	<b>AUC in standard curve</b>	<b>Ratio (nm)</b>
Standard 1	.15	10.000	536537 D <sub>2</sub>	.482
	.05	11.700	1114001 D <sub>3</sub>	
1	.15	10.033	358466 D <sub>2</sub>	.007
	.10	11.266	50772764 D <sub>3</sub>	
2 1:10 dilution	.15	9.600	819134 D <sub>2</sub>	.051
	.20	11.100	15911161 D <sub>3</sub>	
3 1:20 dilution	.15	9.450	330467 D <sub>2</sub>	.071
	.30	11.016	4629098 D <sub>3</sub>	
4 1:40 dilution	.15	9.716	85395 D <sub>2</sub>	.080
	.40	11.216	1060833 D <sub>3</sub>	

**Table 2.4 Proposed and Actual Vitamin D Content in Spray Dried Complex**

Proposed Vitamin D ( $\mu\text{g/L}$ )	Actual Vitamin D ( $\mu\text{g/L}$ )
0	0
10	10.2
20	20.3
40	40.8

**Table 2.5 Rat Chow: Vitamin D Content**

<b>Diet Type</b>	<b>Company Specification</b>	<b>HPLC Analysis</b>
AIN-93G Purified Rodent Diet	400IU/g 0.25g/kg	0.32 mg/kg
Deficient Diet	0g/kg	0.11 mg/kg

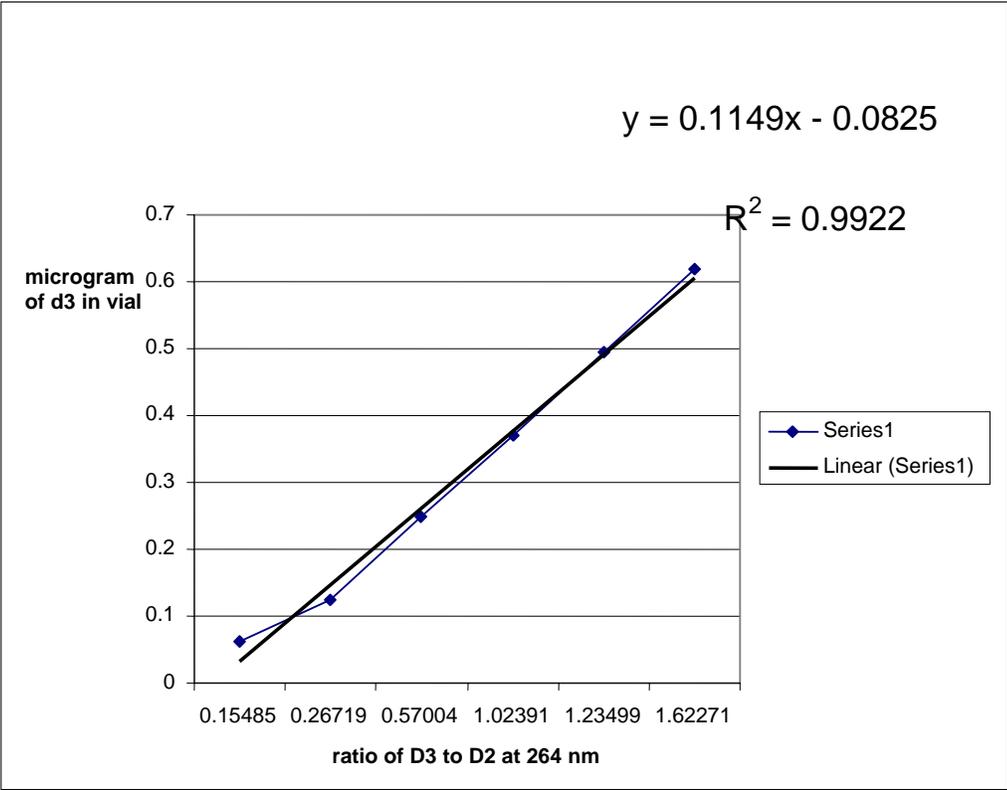


Figure 2.3 – Vitamin D Standard Curve

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CHAPTER 3

**THE BIOAVAILABILITY OF VITAMIN D AND CALCIUM IN RATS**

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### 3.1 – Abstract

Calcium and vitamin D play a critical role in the prevention of metabolic diseases including osteoporosis, osteomalacia, and rickets. Epidemiological research indicates that average intake of these nutrients is well below the RDA, and greater intake has been correlated to a reduction in fractures, prevention of osteoporosis, and increased bone mass. The objective of this study was to test effects of a water-soluble form of vitamin D and calcium chloride as fortifiers for an aqueous sports drink solution with a rat bioavailability assay.

A water-soluble vitamin D fortifying ingredient was prepared as a spray-dried complex with bovine beta-lactoglobulin. Vitamin D content of the complex was assessed by HPLC. Flavored beverages were formulated with various ratios of calcium and vitamin D in a 4x4 factorial design. Female Wistar rats were housed under incandescent lighting and randomly divided into the treatment and control groups. After a 4-week depletion phase, rats were given specialized drink formulations and low calcium, low vitamin D diet for an additional six weeks. Blood and femur bones were removed for further analysis. Serum vitamin D was measured by ELISA.

Results demonstrate that fortified drink solutions could be accurately formulated to contain calcium chloride at 0, 1, 2 and 2.5 g Ca/L with palatability to rats. The vitamin D content of the drinks was formulated to be 0, 10, 20, and 40 µg/L. Serum vitamin D was significantly greater ( $p < .0001$ ) in rats receiving the vitamin D-fortified drinks.

Water-soluble vitamin-D can be used to fortify aqueous products with this fat-soluble vitamin to help facilitate the uptake of calcium. Regular consumption of flavored sports drink

fortified with calcium and vitamin D may significantly increase dietary calcium and vitamin D intake.

### **3.2 – Introduction**

Vitamin D plays a critical role in the homeostatic control of calcium and phosphorus. Thus, adequate intake of vitamin D increases the efficiency of intestinal calcium absorption which facilitates skeletal development (Holick, 2005). The purpose of this study was to examine the effects of a water-soluble form of vitamin D and calcium chloride as fortifiers for an aqueous sports drink solution with a rat bioavailability assay. Calcium and vitamin D play a critical role in the prevention of metabolic diseases including osteoporosis, osteomalacia, and rickets. Epidemiological research indicates that average intakes of these nutrients are well below the RDA, and greater intake has been correlated to a reduction in fractures, prevention of osteoporosis, and increased bone mass (Bischoff-Ferrari, 2006).

Moreover, insufficient intake of vitamin D has led to the re-emergence of vitamin D dependent rickets in some US populations, especially the darkly pigment communities (Nield, 2006). Due to the re-emergence of rickets and the deficient intake of vitamin D, experts have stated that current recommendations for vitamin D are too low. Therefore, these experts are recommending increasing the RDA values for vitamin D (Hathcock, 2007). Current randomized supplementary trials utilizing the current RDA for vitamin D, 400 IU, have not shown sufficient reduction in fracture risk (Vieth, 2007). On the other hand, other research studies have shown supplementation about the current RDA yielded a decrease in fractures (Holick, 1998; Hollis, 2004; Vieth, 2007). These research studies also lay the framework for more fortification of various food products with calcium and vitamin D.

### 3.3 – Materials and Methods

Female Wistar rats (Charles River Laboratory, Raleigh, NC) were individually housed under incandescent lighting with a 12- hour light/dark cycle. Rats were randomly divided into 16 treatment groups and 4 control groups. Immediately after arrival five rats were sacrificed for baseline data. The remaining rats were made deficient in vitamin D for 4 weeks, by feeding a modified AIN-93G diet with normal calcium (5.0 g Ca/Kg) level and lower vitamin D (0.11g/kg), as analyzed after the study concluded. After completion of the reduction phase, an additional five rats were sacrificed and serum was collected and stored for later measurement of 25-OH D<sub>3</sub> by radioimmunoassay (DiaSorin, Stillwater, MN).

A 4X4 factorial design was utilized to divide the animals in subgroups for the drinking water supplementation. The concentrations bracketed our estimate of intake from dietary normal AIN 93G diet, equivalent to 2.88 g Ca/L and 14.4 µg vitamin D<sub>3</sub>/L. These values indicate the estimated daily requirement (EDR) for calcium and vitamin D respectively, based upon previous research conducted in our laboratory. The rats received the fortified water for 6 weeks. The vitamin D content of the sports drinks were formulated to be 0, 10, 20, or 40 µg/L. Moreover, the calcium content of the drink was initially 0, 1, 2, 3.0 g/L as CaCl<sub>2</sub>; however early in the study the rats with the highest of amount of CaCl<sub>2</sub> would not consume the beverage. Therefore the calcium concentrations were altered to 0, 1, 2, 2.5 g/L as CaCl<sub>2</sub> which increased the palatability to the animals (Table 3.1). Fruit flavor and non-caloric sweetener were added to increase palatability. For the duration of the vitamin D repletion stage, animals' drink and food intake was recorded and weight was obtained twice

per week. Rats were sacrificed with a ketamine (650 mg/100 g body weight) + xylazine (140 mg/100 g body weight) anesthetic to achieve unconsciousness, followed by exsanguination. Blood was removed by cardiac puncture using 10-ml syringes and 18-GA needles and serum vitamin D was determined by ELISA (IDS Inc., Fountain Hills, AZ). In addition, the left femurs were weighed and measured. The right femurs were removed, ashed, and analyzed for calcium content.

**Table 3.1 – Drink Formulation: Fortified Flavored Sports Drink**

[Ca] (g/L)/ [Vitamin] D µg/L	0	1.0	2.0	2.5
0	A = 5 rats	B = 5 rats	C = 5 rats	D = 5 rats
10	E = 5 rats	F = 5 rats	G = 5 rats	H = 5 rats
20	I = 5 rats	J = 5 rats	K = 5 rats	L = 5 rats
40	M = 5 rats	N = 5 rats	O = 5 rats	P = 5 rats

**3.3.1 – Bone Assay for Calcium Analysis**

All work pertaining to assay of bone was conducted under the fume hood. Prior to utilization of collected bones, test tubes, glassware and crucibles were acid-washed to remove all foreign material and minerals. The left femur bones were cleaned manually by removing accessible excess tissue. The femurs were soaked overnight in a test tube

containing 100% ethanol; solvent was discarded then the bones were soaked in chloroform overnight. This process was conducted to ensure that all lipid tissue material was removed from the femur bones. The hazardous material was discarded and the bones air dried for approximately five minutes under the fume hood.

The bones were placed in pre-weighed ceramic crucibles that had been previously acid washed in 1N HCl and baked in the furnace at 700 °C for 2-3 hours and cooled in the desiccators overnight. The bones were placed in the muffle furnace at 100 °C for 8-12 hours to remove excess moisture. The bones were placed in desiccators to cool. The dry weight was obtained from weighing the crucibles after cooling. The crucibles were placed back into the muffle furnace at 649 °C for 24 hours, and cooled 8 hours. The crucibles containing the ash were reweighed.

The ashed material was transferred to a test tube and dissolved in 10 ml of 3 N HCl and diluted 1:1000 using 0.5% lanthanum in 0.1 N HCl. Atomic absorption spectrophotometry (Perkin Elmer Model 3100, Norwalk, CT) was utilized to obtain calcium content from the ashed bone.

### **3.3.2 – Serum Analysis for Calcium**

At the point of sacrifice, blood samples were collected in 4 ml Vacutainer tubes, coated with clot activator. The samples were allowed to rest for 30 minutes and centrifuged at 760 x g for 15 minutes. Centrifugation allowed for the separation of serum from the cells. The serum was collected in amber vials and stored in -20°C until analyzed. At the time of analysis the amber vials were placed in a room temperature water bath to facilitate defrosting of the contents. The serum samples were diluted 1:50 using 0.5% lanthanum in 0.1N HCl.

The calcium content was determined by Perkin Elmer Model 3100 (Norwalk, CT) atomic absorption spectrophotometer.

### **3.3.3- Analysis for Serum 25-OH Vitamin D<sub>3</sub>**

An Octeia 25-Hydroxy Vitamin D Elisa Kit (IDS Inc., Fountain Hills, AZ) was purchased to analyze serum 25-OH vitamin D. This kit is approved by the Food and Drug Administration (FDA) for analyzing 25(OH) D status in humans. We verified reactivity in rat serum in a preliminary experiment.

The labeled serum samples were removed from -20°C storage and placed in a room temperature water bath for 2-3 minutes. The protocol followed was in accordance with the manufacturers' product insert. Polypropylene test tubes were labeled for each calibrator, control and sample. Twenty-five µl of each calibrator, control, and sample were added to the appropriate test tube. One ml of 25-D biotin solution was added to all the test tubes. Each test tube was vortexed for approximately 10 seconds. Two hundred µl of each diluted calibrator, control and sample were added to the appropriate well of the antibody-coated plate in duplicate. After the samples were added to the plate, the lid was placed on the plate and sealed in a plastic bag. The plate was incubated at 18-25°C for 2 hours. Following, the incubation period the plate wells were washed manually with 250 µl of buffer solution provided. The buffer solution was decanted and the procedure repeated twice. The plate was inverted firmly on absorbent tissue to remove excess fluid between washings. Two hundred µl of enzyme conjugate was added to each well. The lid was placed upon the plate again and incubated at 18-25°C for 30 minutes. The manual wash step was repeated. After the plate was firmly inverted to remove excess buffer solution, 200 µl of tetramethylbenzidine

substrate was added to each well using a multichannel pipette. The plate was again sealed and incubated at 18-25°C for 30 minutes. One hundred µl of 0.5 M HCl was added to each well with a multichannel pipette to stop the reaction. Within 30 minutes of adding the 0.5 M HCl the absorbency was measured at 450 nm using a microplate reader (Thermo Electron Corporation, Vantaa, Finland). The absorbances of the samples were compared to the standard curve data obtained from use of the calibrators.

### **3.3.4 – Bone Strength Test**

Bone strength has been correlated with bone mass and bone quality (Iwamoto, 2006). Mechanical properties of the rats' femurs were determined with an Instron Universal Testing Instrument (Model 1122 Instron, Canton, MA).

The right femurs were utilized for this analysis, after the bone was dissected from the body and were prepared for the mechanical strength test. The preparation procedure included removing visual soft tissue and muscle from the bone. The bones were individually sealed in plastic bags and labeled and stored at -4°C until needed for strength testing. The femurs were thawed and three measurements (mid width, joint width, and length, all measured in mm) were ascertained with electronic calipers, and the three point bending test was performed. Due to the irregularity in bone structure, bone diameter was taken three times and averaged. For the compression test, the femur bones were placed on two adjustable fulcra, 1.9 cm apart and force was applied to the middle shaft portion at a constant speed 5 mm/min perpendicular to the longitudinal direction of the bone's axis. The maximum shear stress at fracture was the indication of overall bone strength.

### **3.4 – Statistical Methods**

SAS software (Cary, NC) was used for the statistical analysis of the data. Moreover, this software was employed to determine the correlation between calcium and vitamin D in water, calcium and vitamin D in food to other dependent variables. Correlation coefficients for linear regression ( $r^2$ ) were determined. Comparative analysis was conducted between various treatment groups.

### **3.5 Results**

#### **3.5.1- Calcium and Vitamin D Intake**

Calcium intake for the 6- week repletion period was calculated for food and water (weights of offering-refusals on weekly basis) x calcium content in food and water. Groups R and T received the positive control diet and tap water. Group D consumed the greatest amount of calcium via beverage and diet consumption. Furthermore, the animals in the group D were able to consume an equivalent amount of calcium via beverage and diet when compared to the positive control (feed only). The calcium via food intake remained constant for all treatment groups. The highest mean calcium intake from water was seen in treatment groups B, C, and D (Figure 3.1).

Vitamin D intake for the 6-week repletion period was calculated for water (weights of offering-refusals on weekly basis) x vitamin D content in water. Groups A, B, C, and D had no vitamin D from fortified sports drink (Figure 3.2). However, as illustrated in Figure 3.3 the HPLC graph shows that vitamin D was contained in the vitamin D deficient diet in the amount of 0.11 mg/kg.

### **3.5.2- Serum Calcium, Serum Vitamin D and Serum Protein**

Serum calcium was analyzed by a 2-way ANOVA with calcium and vitamin D intakes as main effects. Data among all treatment groups were not significantly different from each other. Serum vitamin D in all treatments was adequate to compensate for low Ca intake. The fact that serum Ca did not change can also be used as the explanation for no treatment effects that will be discussed as it related to bone mass or bone Ca (Figure 3.4).

As illustrated in Figure 3.5, serum 25-OH D<sub>3</sub> was analyzed by competitive ELISA (DiaSorin, Stillwater, MN). After the depletion phase the animals' serum 25-OH D<sub>3</sub> values were reduced. Serum 25-OH D<sub>3</sub> in rats feed control and low vitamin D<sub>3</sub> diets for 6 weeks were 0.4 µg and 0.6µg. Data were analyzed by 2-way ANOVA with Scheffe's post hoc test. Groups receiving no Ca were significantly different from the other groups.

Serum protein was significantly different among groups with various levels of vitamin D intake (Figure 3.6). The serum protein concentration of the rats increased as the amount of vitamin D increased. Increased vitamin D supplementation correlated with increased serum protein. Serum 25-OH D<sub>3</sub> in rats feed control diet for 4 weeks was 55.24 ± 15.24 nmol/L. Serum 25-OH D<sub>3</sub> in rats feed low vitamin D<sub>3</sub> diets for 6 weeks were 91.15 ± 9.26 nmol/L.

### **3.5.3- Bone Parameters and Correlations**

As illustrated in Table 3.1, the final mean body weights were not significantly different among all groups. Bone ash weight was derived from drying and ashing the femur bones of the rats. The average ash weights were not significantly different among the groups.

Additionally, beverage intake was observed. Water consumption was greatest among those groups whose water was composed of no or lowest amount of CaCl<sub>2</sub>. The percent of Ca from the ashed bones were not significantly different. The total intake of Ca from fortified beverages does not correlate with percentage of Ca in bone (Figure 3.7). Moreover, the concentration of vitamin D and calcium did not correlate with the bone parameters assessed (Figure 3.8).

### **3.6 – Discussion**

The purpose of this study was to examine the effects of a water soluble form of vitamin D and calcium supplementation via an aqueous solution on various bone parameters in Wistar rats fed a vitamin D deficient diet. However, the vitamin D deficient diet was analyzed and found to contain vitamin D. These findings may have played a significant role in the results of bioavailability assay, because the baseline vitamin D intake from diet, although only one third as high as control diet, provided more vitamin D than the water in most treatment..

Calcium and vitamin D supplementation in a vitamin D deficient diet did not result in a stimulated increase of food or beverage intake as seen in other supplementation studies (Iwamoto et al., 2003). Supplementation with the fortified aqueous solution containing calcium and vitamin D did not result in a significant difference in live weight (Walter et al., 2000), contrary to other studies (Major et al., 2007). Moreover, supplementation with the fortified beverage had no profound effect on serum calcium. The low calcium intake via food and beverage may have resulted in an increased serum PTH and stimulated more efficient intestinal calcium absorption (Bronner, 2003, Heaney et al., 1997; Iwamoto et al.,

2003). This phenomenon may also have facilitated no significant difference associated with bone strength and length. Uchikura (1999), reported in female Wistar rats that bone mass peaked at 10-12 months. Therefore, this study's duration may have not allowed for unit bone mass peak in this particular species. Additionally, after the first 3 days of treatment staff observed that the animals with the highest concentration of CaCl<sub>2</sub> and vitamin D were not consuming the beverage. The animals were placed on tap water for the remaining four days to rehydrate the animals. On the second week of the repletion study the refusal group received a new formula with a reduced amount of CaCl<sub>2</sub>. The animals responded positively to the new formula. These animals received one week less of the fortified beverage than the other groups.

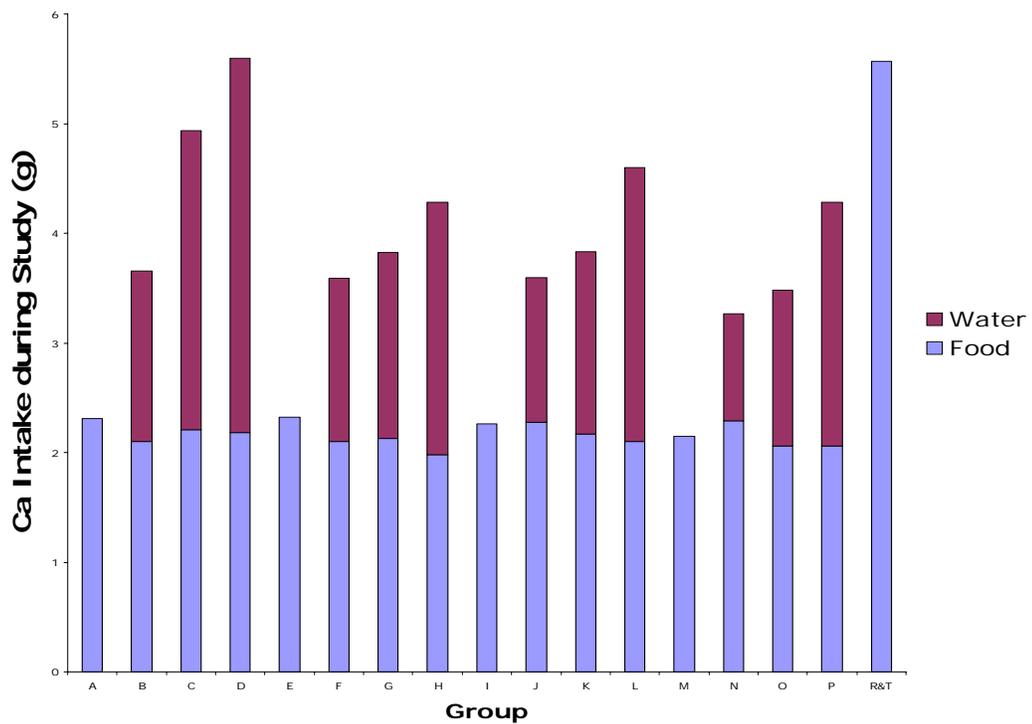
Prevention of bone loss via supplementation with calcium and vitamin D was evident. The results of the present study suggest that calcium and vitamin D work synergistically to maintain bone mass in animals, as similarly seen in human studies (Boonen et al., 2006; Bowen et al., 2004).

Data from the present study clearly illustrate that calcium chloride and a soluble form of vitamin D could be used as fortifiers of an aqueous solution. Furthermore, supplementation with calcium and vitamin D was able to maintain serum calcium and vitamin D levels to prevent negative impact on bone mass. Sufficient amounts of calcium and vitamin D via the diet are important to prevent degenerative bone diseases. Nevertheless, there is still limited research that investigates the use of a water soluble form of vitamin D as a fortifier. Currently calcium and vitamin D are used as fortifiers in dairy

products; however the water-soluble form of vitamin D and calcium chloride could be used in multitude of food applications, even in the absence of fat.

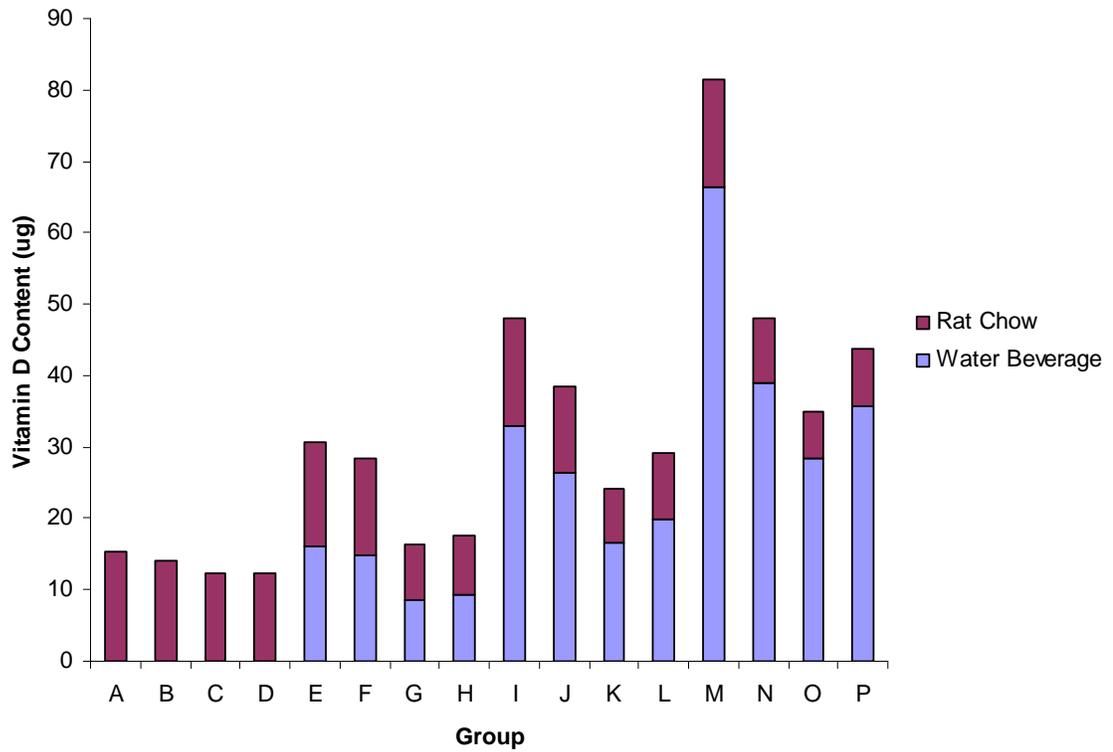
### **3.7 - Conclusion**

A water soluble form of vitamin D can be used to fortify aqueous products to improve vitamin D status and help facilitate the uptake of calcium. Moreover, in this experiment regular consumption of flavored sports drink fortified with calcium and vitamin D was shown to significantly increase dietary calcium and vitamin D intake. Even though the intakes varied significantly, there were no significant effects on calcium concentration or bone parameters. HPLC analyses of diet and serum 25(OH) vitamin D suggests that the rats all had enough vitamin D to adapt to low calcium intake. Nonetheless, the aqueous vitamin D significantly increase serum 25-OH D<sub>3</sub>, a primary indicator of vitamin D status, compared to groups that had no vitamin D in the drinking water.



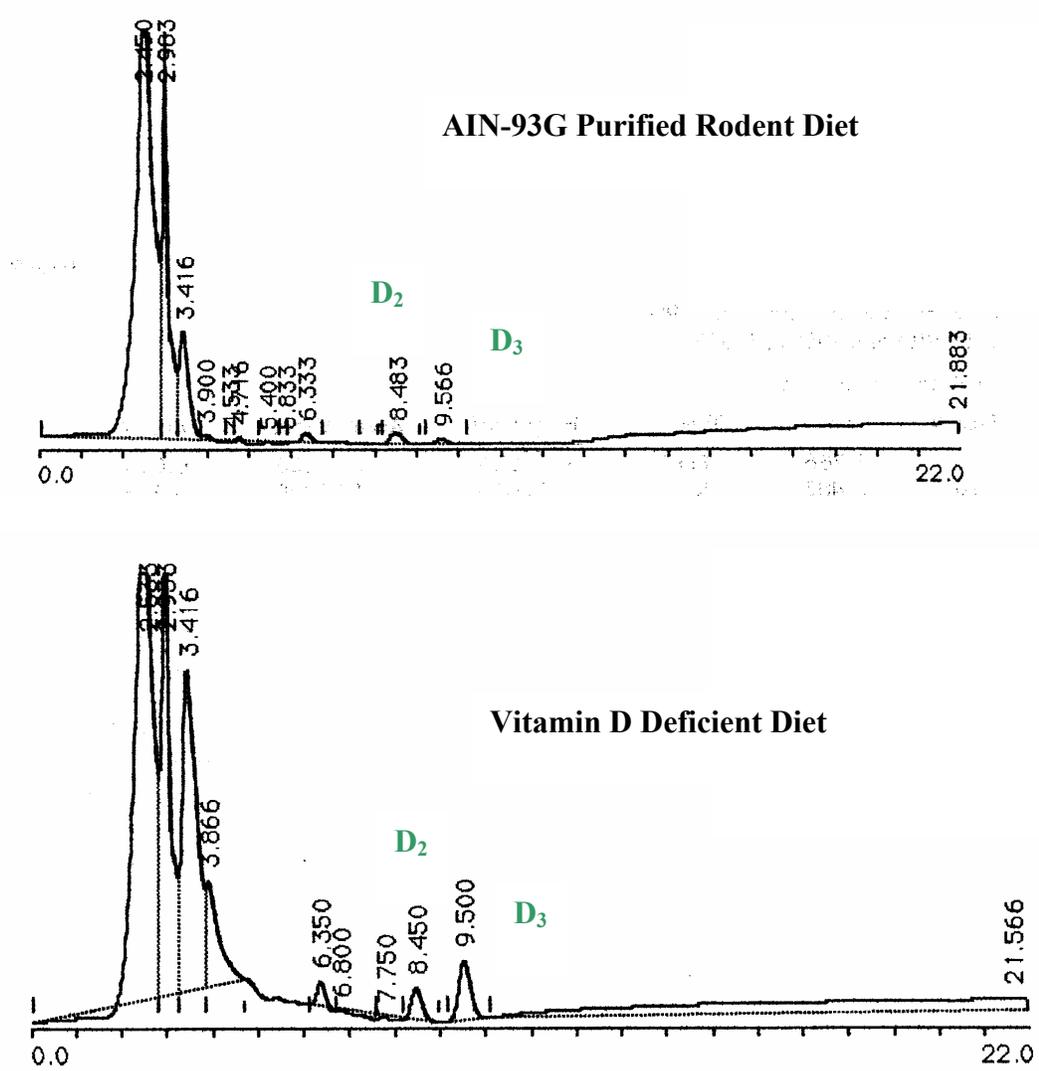
**Figure 3.1- Calcium Intake**

Calcium intake for the 6-week repletion period was calculated for food & water (weights of offering - refusals on weekly basis) x Ca content in food & water. Groups R and T received the positive control diet and tap water.



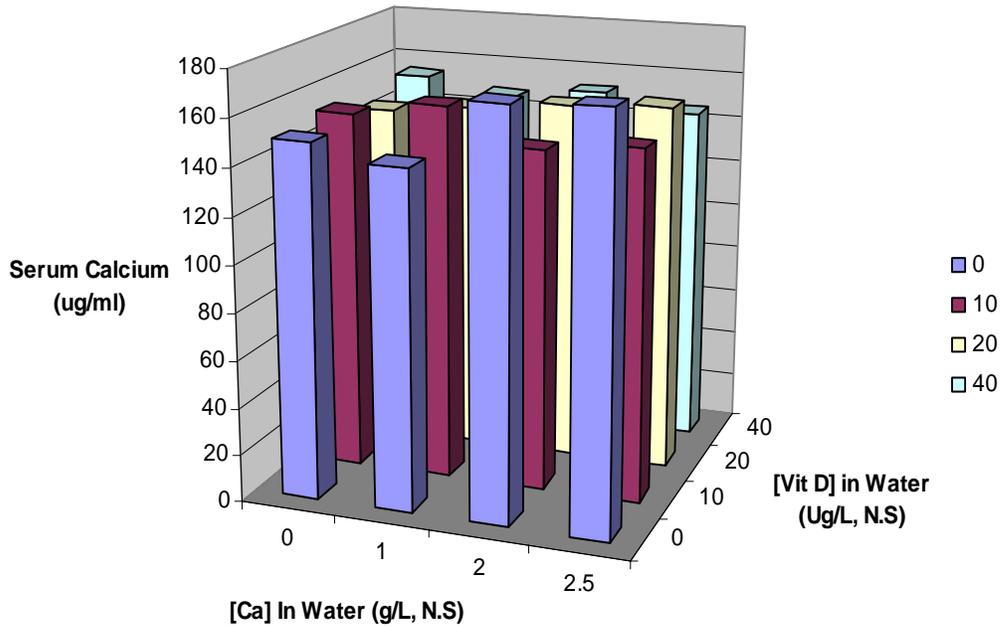
**Figure 3.2 – Vitamin D Intake**

Vitamin D intake for the 6-week repletion period was calculated for water (weights of offering - refusals on weekly basis) x vitamin D content in water.



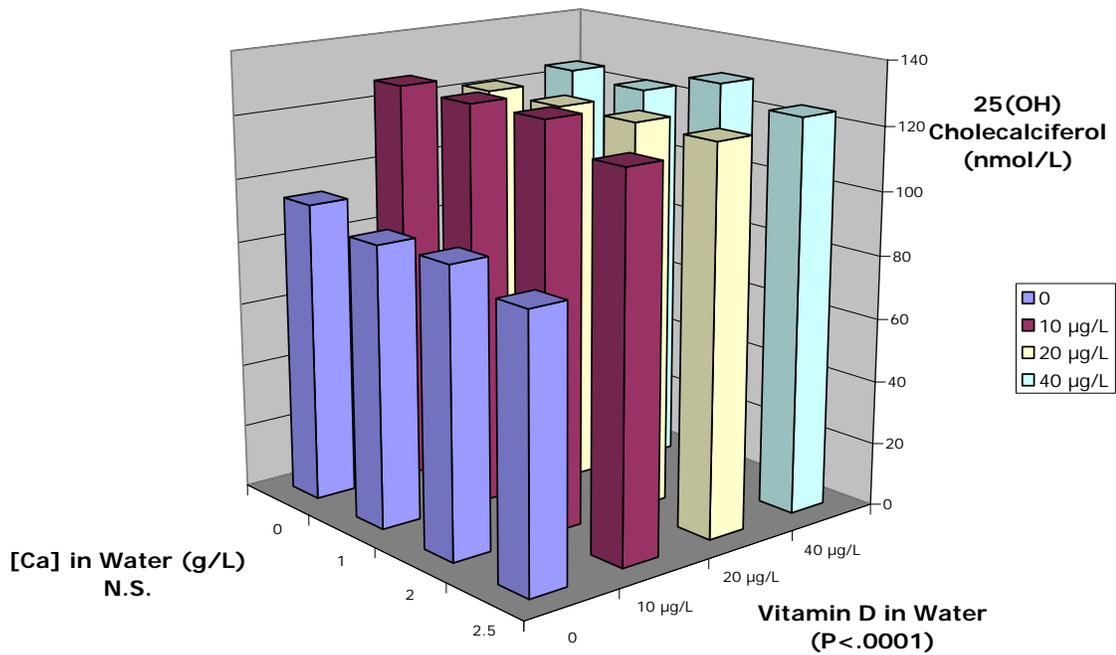
**Figure 3.3 Representative Chromatograms Illustrating Vitamin D Deficient Diet Compared to Normal Diet**

HPLC analysis verified the presence of vitamin D in the deficient rat chow.



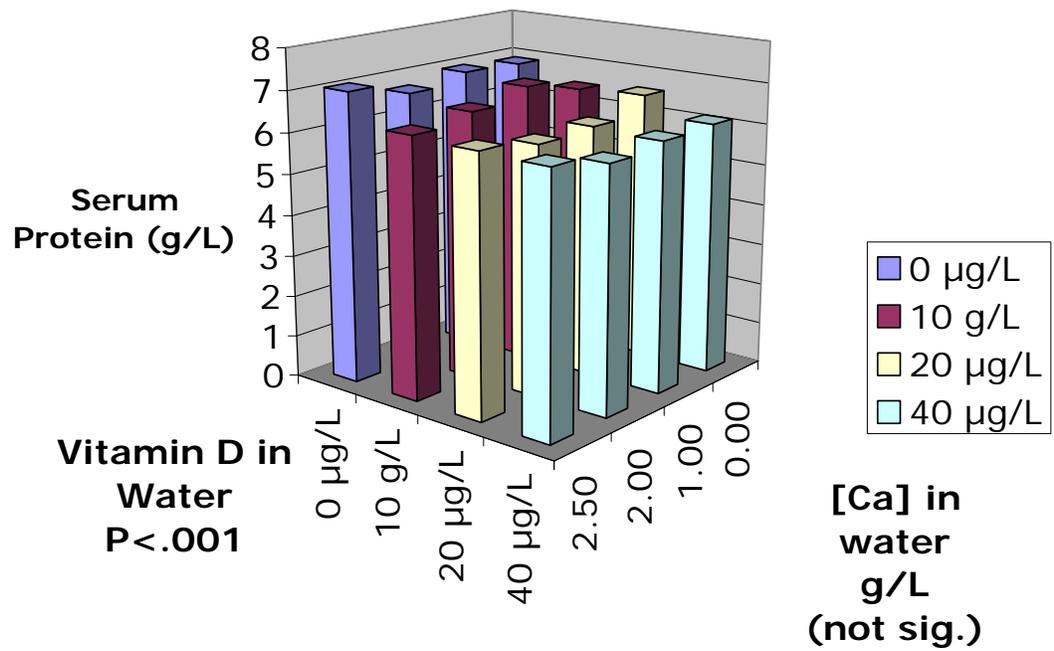
**Figure 3.4 – Serum Calcium**

Serum calcium (Ca & Vit D intakes) was analyzed by a 2-way ANOVA. Data show no significant differences between groups. Serum vitamin D in all treatments was adequate to compensate for low Ca intake. The fact that serum Ca did not change can also be used as the explanation for no treatment effects on bone mass or bone Ca.



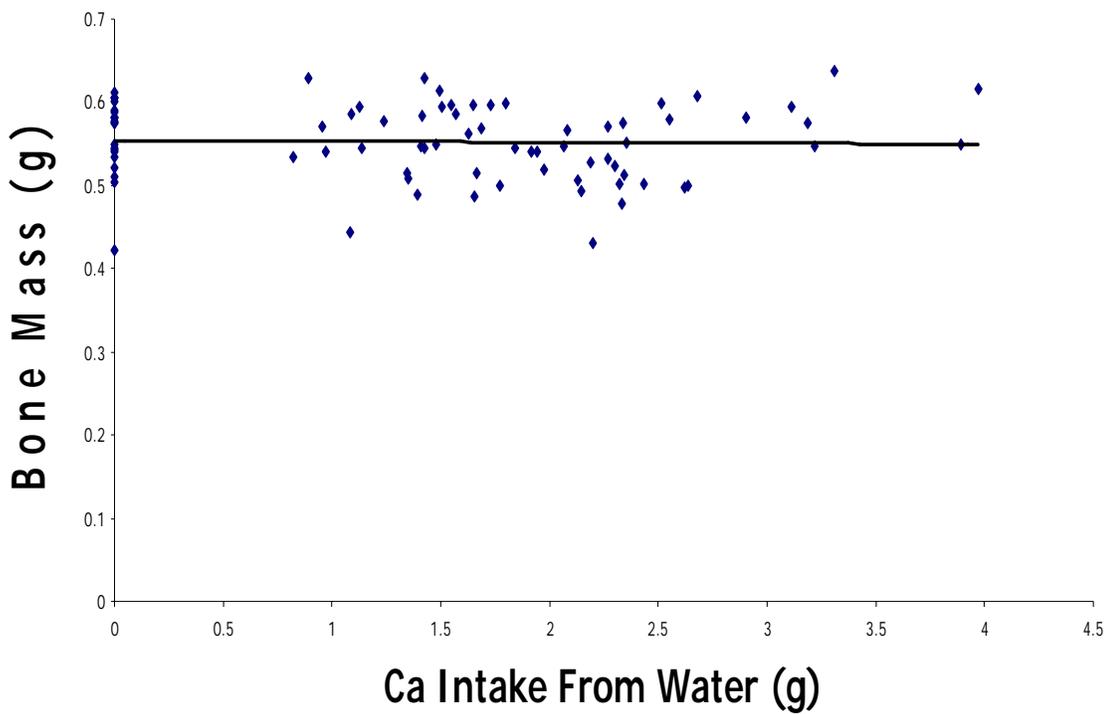
**Figure 3.5- Serum 25-OH Vitamin D**

Serum 25-OH vitamin D was analyzed by competitive ELISA (IDS Inc., Fountain Hills, AZ). Data were analyzed by 2-way ANOVA with Scheffe's post hoc test. Group receiving no Ca was significantly different from the other groups.



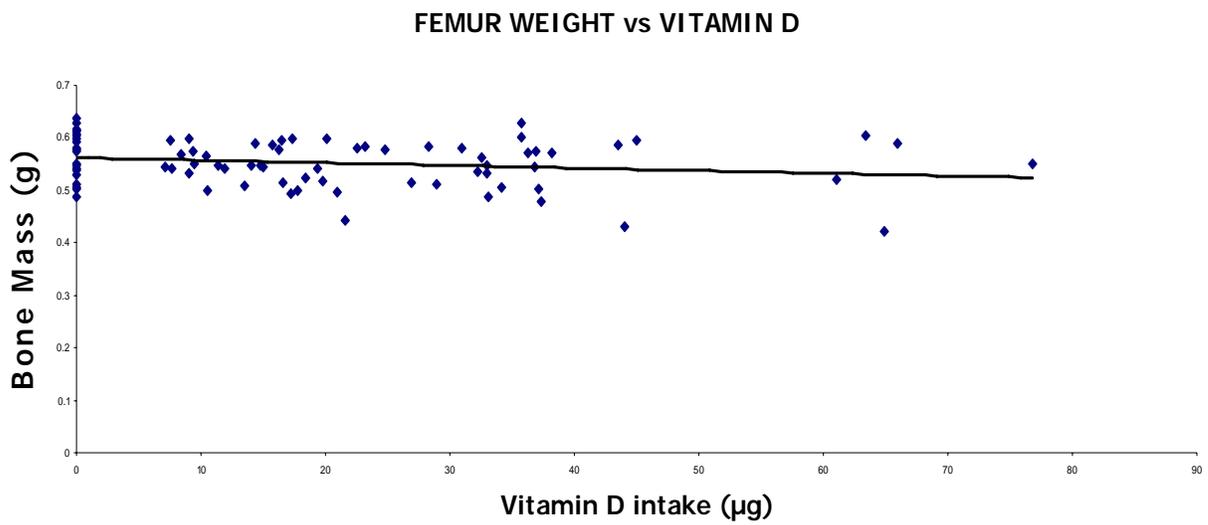
**Figure 3.6 – Serum Protein**

Serum protein was not significantly different among the various treatment groups. However, high concentration of vitamin D correlated with increased serum protein.



**Figure 3.7 – Correlation between Bone Mass and Calcium Intake**

Femur weights were not significantly affected by the ratio of calcium intake, regardless of vitamin D content in water. Low calcium intake of bone was compensated for by vitamin D. The slope of the regression line was not significantly different from zero.



**Figure 3.8 – Correlation between Bone Mass and Vitamin D Intake**

Femur weights were not significantly affected by the vitamin D content in water. The slope of the regression line was not significantly different from zero.

**Table 3.2 – Bone Parameters**

<b>Group</b>	<b>Final Group weight (9/05/05)</b>	<b>Ashed bone weight (g)</b>	<b>Total Drink Intake (ml)</b>	<b>Total Food Intake (g)</b>	<b>% Ca in Ashed bone</b>	<b>Mid width (mm)</b>	<b>Joint width (mm)</b>	<b>Length (mm)</b>
<b>A</b>	281.03	0.29	1,696.40	659.98	43.65	2.46	5.62	31.98
<b>B</b>	255.73	0.29	1,561.23	599.48	55.42	2.14	5.53	33.82
<b>C</b>	263.48	0.30	1,362.82	631.12	43.24	2.52	6.08	32.30
<b>D</b>	283.88	0.31	1,365.88	622.53	49.34	2.63	6.17	32.82
<b>E</b>	286.73	0.28	1,600.54	664.22	45.47	2.44	6.02	31.90
<b>F</b>	266.95	0.28	1,483.50	600.74	47.34	2.52	5.86	31.48
<b>G</b>	274.38	0.30	850.08	607.32	51.70	2.42	5.76	32.16
<b>H</b>	259.25	0.28	921.34	565.26	57.28	2.72	5.94	31.98
<b>I</b>	290.30	0.27	1,646.86	645.95	52.62	2.78	6.32	31.10
<b>J</b>	280.43	0.27	1,318.70	651.12	46.02	2.66	6.16	31.56
<b>K</b>	250.30	0.30	832.46	619.70	46.50	2.52	6.04	31.76
<b>L</b>	270.20	0.29	998.94	599.92	52.29	2.60	6.00	31.10
<b>M</b>	286.23	0.26	1,660.15	614.10	63.15	2.94	6.06	31.68
<b>N</b>	285.00	0.29	976.94	654.95	52.33	2.84	6.62	34.62
<b>O</b>	216.83	0.26	709.72	588.59	46.03	2.68	5.58	30.83
<b>P</b>	259.75	0.28	889.58	589.20	49.26	2.70	6.42	32.90

Calcium and vitamin D intake from the fortified beverage and diet had no significant effect on bone parameters.

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