

## **Abstract**

ELEDAH, JULIE IFEOMA. Calcium Chloride-Fortified Beverages: Threshold, Consumer Acceptability and Calcium Bioavailability (Under the direction of Dr. Jonathan Allen and Dr Maryanne Drake).

In the U.S., 90% of women, 60% of men, and over 50% of children consume less than the RDA for calcium. Calcium intake through dairy product consumption is declining as other beverages replace milk. Calcium fortification of water or flavored waters could increase calcium intake without impacting caloric intake.

The nutritional significance of calcium in bottled water is often questioned. The content in a serving for many brands of spring water is usually less than 2% of the Daily Value. However, mineral water and fortified water could have calcium contents high enough to show on a Nutrition Facts Label. The objectives of this study were to determine the sensory threshold of calcium chloride in water and flavored water, design an acceptable calcium-fortified beverage, and determine the bioavailability of calcium from calcium chloride-fortified drinking water in vitamin D-deficient rats.

Mineral content was determined for nine commercial bottled and flavored waters by atomic absorption spectrophotometry. Sensory detection thresholds of calcium chloride were determined in deionized water (DI), tap water, berry flavored water, and a sports beverage formulation using a five series ascending forced choice analysis. Ascending calcium chloride concentrations in the fortified drinks were evaluated by 30 panelists in duplicate trials. Ninety-eight consumers evaluated acceptability of de-ionized and flavored water with and without added calcium chloride.

Vitamin D-deficient diets of rats had either low (0.2%) or normal (0.5%) calcium content. After a 2-wk depletion phase, drinking water fortified with calcium chloride calculated to provide 0, 0.25, 0.5 or 1.0 times the usual calcium intake (estimated at 2.88 g/L) from diet was provided. Additional groups also had water fortified at 2 x usual dietary intake, but did not complete the study because of low feed and water intake. Some groups also had a water-soluble form of vitamin D added to the water.

Calcium concentration of the nine commercial products measured varied from 0.3 mg/L to 116 mg/L. Sensory thresholds for calcium chloride in drinks were: flavored water ( $857 \pm 8.9$  mg/L) > sport drinks ( $844 \pm 9.8$  mg/L) > DI- water ( $101 \pm 3$  mg/L) > tap water ( $93.5 \pm 3$  mg/L), respectively. (On the basis of Ca, thresholds were flavored water ( $7.72 \pm 0.08$  mM) > sport drinks ( $7.60 \pm 0.09$  mM) > DI- water ( $0.91 \pm 0.01$  mM) > tap water ( $0.91 \pm 0.01$  mM), respectively.) Consumer acceptability scores were not different for water or flavored water with and without added calcium chloride (70 mg/L and 700 mg/L, or 0.63 and 6.31 mM, respectively) ( $p > 0.05$ ).

In the bioavailability study, the final serum vitamin D level indicated that the vitamin D in water was poorly available to the rats. Results showed that groups fed low dietary calcium and not supplemented with calcium in water had lower bone weight, bone ash weight, and bone ash calcium percentage, than did the rats supplemented with calcium in water. The deficient rats with no calcium and low vitamin D and those with highest Ca (2.88 g/L) in water all had lower final body weight than the control group (Ca and vitamin D in diet), and the groups with moderate water Ca (1.44 g/L). Body weight

was correlated with food intake. For groups with 0.5% dietary Ca, bone weight, size, Ca, or breaking strength was not related to the Ca or vitamin D intake from water.

Calcium content in flavored drinks or water can be increased with calcium chloride without impacting acceptability. Regular consumption of calcium fortified water can significantly reduce the effect of low dietary Ca intake on bone growth and mineralization. The fortification of water has little additional effect on bones when dietary calcium is adequate.

**Calcium Chloride-Fortified Beverages: Threshold, Consumer  
Acceptability and Calcium Bioavailability**

by  
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## **BIOGRAPHY**

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While at BASF, the author decided to return to school in 2002. Julie pursued a M.S. in Food Science at NC State University under the guidance and direction of Dr. Jonathan Allen.

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CHAPTER 1  
**LITERATURE REVIEW**

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## **1.1 Preface**

Calcium and vitamin D are associated with prevention of several diseases including osteoporosis, osteomalacia and rickets. Adequate amounts of these nutrients have been shown to help reduce fracture, prevent osteoporosis and increase bone mass.

Sunlight is a good source of vitamin D; an exposure of 15-20 minutes of sunlight can provide the daily requirement for vitamin D. Certain situations prevent people from getting vitamin D to meet their daily requirement, for example, elderly who are institutionalized.

Calcium is an essential nutrient for bone and teeth mineralization. Frequency of low consumption of calcium is increasing because people are consuming more low-fat products. Milk is the predominant source of calcium in the diet, but it is not usually categorized as a low-fat product. Fortification of calcium and vitamin D is not a novel idea, but more new products are being fortified with calcium and vitamin D.

Bottled water consumption is increasing, because consumers are aware of the health benefit associated with water. Fortifying water with nutrients, sweetener, and flavor is likely to increase bottled water consumption even more. Taste is very important to consumers, so enhancing the taste of a fortified product is important for the acceptability of the product.

In conclusion, increasing water consumption is beneficial to consumers. Fortifying these products with nutrients and flavor has the potential to increase consumption and at the same time increase nutrient intake.

## **1.2 Introduction**

### **1.2.1–Water**

The most functional food needed and used by the body is water. It is considered the most important food on the planet; an essential nutrient needed to sustain life. But little attention is given to water compared to other nutrients. In the US food pyramid, nutritionists do not include water. South African and the Greek pyramids attempted to include water in their dietary guidelines. The South African dietary guidelines stated “Drink lots of clean, safe water”, while the Greek pyramid has a footnote stating, “Also remember to drink plenty of water” (Burlingame, 2003). The United Nations General Assembly acknowledged 2003 as the International Year of Freshwater ([www.wateryear2003.org](http://www.wateryear2003.org)). This proclamation will increase awareness on the importance of water consumption within governments and the United Nations.

### **1.2.2 – Dehydration**

Insufficient water consumption can lead to muscle spasm, renal dysfunction, and risk of bladder cancer and finally death. Dehydration is defined as the depletion of body water. It can be mild or chronic, leading to various health conditions. Susan Kleiner, owner of High Performance Nutrition listed some of these conditions as follows (Kleiner, 1999):

- ✗ Diminished physical performance
- ✗ Lowered mental performance
- ✗ Impaired salivary gland functions
- ✗ Greater risk of kidney stones
- ✗ Higher risk of urinary tract cancers
- ✗ Increased risk of colon and breast cancer
- ✗ Risk of childhood obesity

According to a survey of American adults conducted in 2000 by Yankelovich Partners for The Rockefeller University and the International Bottled Water Association (IBWA), there are numerous reasons for not drinking adequate water as shown in

Table 1.1

**Table 1.1**

| Reasons for low water consumption | Percentage |
|-----------------------------------|------------|
| Lack of time                      | 21%        |
| Taste                             | 13%        |
| Prefer other beverages            | 12%        |
| Forgetting                        | 10%        |
| Not feeling thirsty               | 8%         |
| No bottle water available         | 4%         |
| No time for hydration break       | 4%         |
| Too much restroom break           | 2%         |

(IBWA: National Fact Sheet. Survey: America's poor drinking habits contradict knowledge of health risk)

### **1.2.3 - Flavored Water**

#### **1.2.3a- Trends and Sales**

In 2002, Americans spent \$7.7 billion dollars on bottled waters, a 10% increase from the previous year, making bottled water the number three commercial beverage, as reported by the Beverage Marketing Corporation (Decker, 2003). But as bottled water becomes more of a commodity purchase, industries are trying to expand the bottled water market by adding vitamins, sweeter, flavor, etc. to water. These additives are setting bottled water brands apart and have increased sales in this beverage segment to \$245 million in 2002 compared to \$20 million in 2000. Consumers perceive this flavored bottled water as a healthful replacement for soft drinks, which have more calories. “Easier to drink” and “helps us drink more water” were some of the positive remarks noted with flavored bottled water (Decker 2003).

### **1.2.4 - Fortified Flavored Water and Sport (Energy) Drinks**

Flavored water is widely accepted among consumers and water fortified with nutrients like calcium and vitamins is more accepted. Some of the fortificants used are not water-soluble and are incorporated into these drinks encapsulated, bound to a protein or as a nanoemulsion, encapsulating a vitamin into a matrix.

According to the Beverage Marketing Corporation, fortified drinks account for approximately 30% of beverages consumed in the United States and approximately 40% if alcoholic beverages are excluded. Demand for energy drinks is also rising as sales doubled to \$275millions in 2001 from \$130millions in 2000. Sales are projected to rise to \$3.1 billion by 2006 (Brown 2002).

### **1.2.5 - Benefits**

The main benefits of consuming flavored water and sport drinks are the ease in drinking, better hydration, and access to vitamins. Some consumers don't drink water because of the bland taste. Marketing a type of water with sweetener, flavor, and vitamins and minerals added and zero-calories can be considered miracle water to the consumer. Increasing numbers of people now prefer these drinks to soft drinks (Decker 2003).

## **1.3 – History of Fortification**

### **1.3.1 - History**

Fortification can be defined as the addition of nutrients to foods (<http://www.fnri.dost.gov.ph/htm/fdforti.htm#ref>). The first fortified food, iodized salt, was introduced in Michigan in 1924 because of goiter, a common disease caused by deficiency in iodine. Soon after the introduction of this fortified food, prevalence of goiter fell from 38.6% to 9%. By the 1930s, iodine deficiency was almost eradicated as a public health problem. During the late 1930s, a need for food fortification was evident as minerals and vitamin deficiency was prevalent in the US population with increasing epidemics of disease such as pellagra, rickets and night-blindness.

In May of 1941, President Roosevelt called a National Nutrition Defense meeting where the now-called Food and Nutrition Board first presented the Recommended Daily Allowances (RDAs). The new committee established different RDA for different

nutrients and also approved fortification of foods like flour, bread, cornmeal, milk, and margarine with thiamin, niacin, riboflavin, vitamin D, and vitamin A.

In the 1990s, the nutritional status in the United States was different from the 1930s or 1940s and food fortification, among other factors, can be credited for such improvement (Backstrand, 2002).

## **1.4 – Calcium**

### **1.4.1 - Calcium source and metabolism**

Calcium, an essential nutrient, is a major component of mineralized tissue for normal growth. More than 99% of the calcium in the body is held in the bones and teeth (Wardlaw 1999). Parathyroid Hormone (PTH), calcitonin, and vitamin D regulate calcium metabolism to maintain serum calcium level. Low intake of calcium will lead to release of calcium from bone, increasing the risk of osteoporosis (Bendich 2001). Calcium levels must be regulated for normal physiological functions like responding to hormones and cell proliferation (Adi et al. 2002, Bendich 2001). Good sources of calcium in the diet are calcium-rich foods like milk, broccoli and cheese or fortified foods like cereal and juice. The recommended dietary allowances for calcium range from 1200-1500 mg/ day in various nations (Guillemant et al. 2002)

### **1.4.2 - Bioavailability and Digestion**

Bioavailability is defined as absorption, transport, and utilization of the nutrient. (Weaver, 1998). One of the main concerns in fortification is bioavailability of added nutrients compared to that naturally occurring in food. Absorption of calcium is a common indicator of bioavailability. Absorption of calcium can be enhanced or inhibited

by several nutritional factors. Nutritional factors that enhance calcium absorption include lactose while fiber, phytate, oxalate, and caffeine inhibit calcium absorption (Heaney, et al 1995).

Absorption occurs in the small intestine (duodenum) because calcium can stay in solution in its ionic state better with pH below 6. The contents from the stomach enters the small intestine at low pH but are then neutralized by bicarbonate released from the pancreas. The active Vitamin D hormone, calcitriol, is needed for calcium absorption within the upper small intestine. Large quantities of calcium are also absorbed in the ileum and jejunum because the food spends a longer time there and passive, paracellular transport pathways are available (Wardlaw1999 and Mann et al, 1998).

### **1.4.3 – Functions**

Inadequate calcium intake is associated with several chronic diseases. The function of calcium for the prevention of these chronic diseases has been extensively studied.

#### **1.4.3a - Osteoporosis and Calcium**

The occurrence of osteoporosis increases dramatically with age. This major health problem becomes more important with increases in life expectancy (Chapuy et al, 1998). More than 99% of the body's calcium is in the skeleton, which serves as a reservoir to help replenish calcium in the blood and soft tissue when calcium is insufficient in the diet (Weaver, 1998). By increasing the mineral density in the body through increases in calcium intake, the risk of fracture may be reduced. Several studies have shown that increasing dietary calcium through fortification and/or other supplementation can increase bone mass (Johnston et al., 1992, Lloyd et al., 1993).

#### **1.4.3b - Kidney Stones and Calcium**

Calcium may protect against kidney stone formation by complexing with oxalate in the gut and reducing oxalate absorption. But calcium supplements must be taken with food in order show this effect (Weaver, 1998).

#### **1.4.3c - Blood Clotting and Calcium**

Several steps are involved in blood clotting and calcium helps with the formation of fibrin from fibrinogen. Prothrombin conversion to thrombin is dependent on the availability of calcium in the blood. Thrombin helps with the formation of fibrin, an insoluble protein that cross-links to form threads that trap blood cells, platelets, and fluid creating a blood clot. Low levels of calcium in the blood will prevent blood clotting (Wardlaw 1999).

#### **1.4.4 – Calcium Fortification**

Awareness of the importance of adequate calcium in our diet is leading to more fortified foods becoming developed. Some of the products fortified with calcium are breakfast cereals, orange juice, milk, yogurt, cheese, ready-to-eat (RTE) meals and vegetables (Gras et. al, 2003). The United States Recommended Dietary Allowances for calcium and optimal calcium intake are as follows:

**Table 1.2: Calcium RDA**

| Group                                  | RDA 1989 <sup>a</sup><br>(mg/day) | Optimal daily intake <sup>b</sup><br>(mg/day) | Adequate Intake 1997 <sup>c</sup> |
|----------------------------------------|-----------------------------------|-----------------------------------------------|-----------------------------------|
| <b>Infants</b>                         |                                   |                                               |                                   |
| Birth – 6 months                       | 400                               | 400                                           | 210                               |
| 6mths – 1yr                            | 600                               | 600                                           | 270                               |
| <b>Children</b>                        |                                   |                                               |                                   |
| 1 – 5 yrs                              | 800                               | 800                                           |                                   |
| 1-3 yr                                 |                                   |                                               | 500                               |
| 6-10 yrs                               | 800                               | 800-1200                                      |                                   |
| 4-8 yr                                 |                                   |                                               | 800                               |
| <b>Adolescents/Young Adults</b>        |                                   |                                               |                                   |
| 11-24yrs                               | 1200                              | 1200-1500                                     |                                   |
| 9-18 yr                                |                                   |                                               | 1300                              |
| <b>Men</b>                             |                                   |                                               |                                   |
| 25-65yrs                               | 800                               | 1000                                          |                                   |
| Over 65yrs                             | 800                               | 1500                                          |                                   |
| <b>Women</b>                           |                                   |                                               |                                   |
| 25-50yrs                               | 800                               | 1000                                          |                                   |
| <b>Men and women</b>                   |                                   |                                               |                                   |
| 19-50 yr                               |                                   |                                               | 1000                              |
| Over 50                                |                                   |                                               | 1200                              |
| <b>Over 50yrs<br/>(postmenopausal)</b> |                                   |                                               |                                   |
| 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 – Sensory Evaluation of Calcium Chloride in Beverages**

As atomic weight increases, bitter and sour tastes of metallic ions increase, but these tastes surpass saltiness. Calcium salt is a complex taste and its property in the overall flavor of food is hard to predict. An inhibitory pattern among the four classical tastes (bitter, sweet, salty, and sour) might be present in calcium salt (Lawless et. al, 2003). This phenomenon is called mixture suppression. For instance, the sourness of an acid in a drink can be easily masked by the sweetness in sugar. The sugar serves two purposes; to provide sweetness and at the same time, decreasing the intensity of sourness (Lawless and Heymann 1999).

Calcium chloride is not the ideal calcium salt to use in food fortification because of its high bitterness ratings. Sweet-tasting compounds like sucrose have been shown to suppress bitterness and even acid (Lawless et. al, 2003).

In a recent study, a calcium chloride solution alone produced a bitter taste. Sucrose, on the other hand, suppressed the bitterness from calcium chloride. Citric acid only suppressed the bitter taste of calcium chloride at higher concentrations of calcium chloride. At this level, calcium chloride taste is perceived as sour and can be easily suppressed by sucrose, but acid slightly suppresses the sweetness. In combination, a sweet compound like sucrose and an acid will suppress the bitter taste of calcium (Lawless et. al, 2003).

## **1.6 - Vitamin D**

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

In 1919, Edward Mellanby discovered Vitamin D during his experiment with rickets (Mehta et al, 2001). It originated from a group of steroid molecules: 9, 10

secosteroids with differentiating side chain structures. It is known for its central role in regulating mineral homeostasis (Leeuwen et al, 2001) especially calcium and phosphorus.

The main sources of vitamin D are through exposure to sunlight or consuming fortified foods (DeLucia et al, 2002), but the synthesis of vitamin D through sunlight in the skin decreases with age due to the decreased capacity of the skin to function normally (Lips 2001). Vitamin D is classified as a prohormone because it is a precursor of an active hormone not consistently derived from diet.

### **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. When ingested through fortified food, vitamin D is absorbed along with the dietary fat in the intestine while sunlight converts 7-dehydro-cholesterol to vitamin D (cholecalciferol). Vitamin D binds to a protein carrier in the blood and is transported to the liver. While in the liver, it is metabolized to 25-OH vitamin D and then further metabolized in the kidney to 1, 25 dihydroxy vitamin D; the active vitamin D hormone, also called calcitriol, which is then transported to target tissues in the body.

### **1.6.3 – Function**

Vitamin D<sub>3</sub> regulates blood calcium by increasing absorption of calcium through the small intestine, reducing calcium excretion through the kidney and regulating calcium deposition in the bones (Wardlaw 1999). It has been demonstrated that vitamin D or its metabolites are fundamental for normal insulin secretion (Cade et al., 1986; Kadowaki et al., 1985). Reports show that the 1,25 (OH)<sub>2</sub> D<sub>3</sub> receptors have been found in the heart

(Simpson, 1983; Walters et al., 1986; Bidman et al., 1991) and may be involved in the role of calcium in cardiac function (Stio et al., 1994).

Vitamin D affects cell growth and differentiation, the immune response (Bouillon et al., 1995), and the brain (Garcion et al., 2002; Eyles et al., 2003). Calcium and phosphorus levels in the plasma are regulated by vitamin D (Holick, 1999). Rheumatoid Arthritis (RA), an inflammation of the joints caused by an autoimmune disorder has been linked to vitamin D. According to a study in Iowa, women who consume the most vitamin D either through diet or supplementation were less likely to be diagnosed with RA (Merlino et al 2004).

Sunlight may supply most of the vitamin D needed in temperate climates, but in certain parts of the world the supply of sunlight is limited. Atmospheric pollution, among other factors, reduces the sun's intensity. In a study done in India, researchers compared vitamin D status of 34 children in an area of high levels of pollution (Mori Gate) to children living in a less pollution area (Gurgaon). The study concluded that children living in the high-polluted area are at risk of developing vitamin D deficiency (Agarwal et al., 2002).

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

Insufficient vitamin D elevates serum parathyroid hormone (PTH), increases alkaline phosphate activities, reduces serum calcium and serum phosphate, and also increases the urinary excretion of calcium, causing an increase in bone resorption. The effect of vitamin D deficiency is termed rickets in children and osteomalacia in adults. Both diseases are common in children and women who usually stay indoors and fully

covered when outdoors. Malabsorption is another factor that may increase the risk of rickets and osteomalacia. In chronic kidney failure, vitamin D activation by 1- $\alpha$  hydroxylase is impaired (Mann et al, 1998).

### **1.6.5 - Vitamin D Fortification**

The main sources of vitamin D are through sunlight, vitamin D-rich food and fortified foods. The vitamin D fortified foods are important for protection of those at risk of insufficient sunlight exposure. Examples of foods currently fortified with vitamin D are milk, cheese (Banville et. al, 2000), margarine, and orange juice.

In 1941, President Roosevelt issued a request to begin fortifying food with vitamin D in order to avoid vitamin D deficiency. This resulted in Vitamin D-fortified milk; other food currently fortified with vitamin D includes yogurts, cereals and nutritional bars (Peregrin 2002).

**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>    | <b>5</b>                                             |
| <b>Children</b>          |                                                      |
| <b>1-13yrs</b>           | <b>5</b>                                             |
| <b>Adults</b>            |                                                      |
| <b>14-50yrs</b>          | <b>5</b>                                             |
| <b>51 to 70yrs</b>       | <b>10</b>                                            |
| <b>Over 70yrs</b>        | <b>15</b>                                            |
| <b>Pregnant Woman</b>    | <b>5</b>                                             |
| <b>Nursing Women</b>     | <b>5</b>                                             |

*Source: U.S. National Academy of Science, 1997*

### **1.7 - Vitamin D receptor (VDR)**

Vitamin D easily disperses into the cell membrane and then transfers through the cytoplasm to the nucleus where it binds to the VDR, a soluble receptor protein. VDR is

ubiquitous in nature and can be seen in many organs including kidney, immune system cells, muscle, heart, etc.

VDR, a transcription factor, is the main nuclear receptor for  $1\alpha, 25(\text{OH})_2\text{D}_3$ ; a physiologically active form of Vitamin  $\text{D}_3$  and also a mediator for all genomic actions of vitamin  $\text{D}_3$  and its analogues (Carlberg et al 2001).  $1\alpha, 25(\text{OH})_2\text{D}_3$  plays a very important role in bone formation, as it is the main regulator of calcium homeostasis (DeLuca et al, 1990). VDR switches on the genes that induce calbindin, a calcium transport protein in the small intestine. As stated, VDR has a great affinity for vitamin D and it also has a DNA-binding site.

Different forms of the VDR have been reported and linked to the hormone's adverse effect on health. The immune function link is the result of the calcitriol's activation of monocytes, its stimulation of cell-mediated immunity, and the suppression of lymphocyte proliferation (Tachi et al, 2003). The regulation of bone metabolism by the VDR is arousing more research interest as a risk factor for osteoporosis (Ferrari et al, 1995). Polymorphism of VDR has raised the risk of breast cancer (Ingles et al, 2000).

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

Adequate calcium intake throughout life is important and different supplementation methods are now available to help increase calcium intake. The synergistic interaction of calcium and vitamin D has helped reduce fracture risk, including hip fracture rate, within 1 year of beginning supplementation (Chapuy et al, 1992). Vitamin D and calcium supplementation combined has been shown to reduce fracture risk in institutionalized elderly French women (Michaëlsson et al, 2003). Osteoporosis in men and women can be reduced significantly when these supplements

are included in diets. Besides osteoporosis, these two nutrients influence other health conditions. For example, calcium helps increase HDL, prevent colon polyps, reduce blood pressure, reduce the reoccurrence of kidney stones and helps with weight loss. Vitamin D on the other hand, may reduce the incidence of some forms of cancer and type-1 diabetes. Synergistically with calcium, it helps reduce tooth loss (Moyad, 2003).

### **1.8.1 - Calcium Chloride/ Vitamin D and Fortification**

Foods fortified with calcium are gaining interest among consumers today. Many people do not meet the RDA for calcium with non-fortified foods. The role of calcium in the prevention of osteoporosis, a condition affecting 20-30 million Americans (Lawless, 1993), is well recognized. According to a 1994 United States Department of Agriculture Continuing Survey of Food adjusted for day-to-day variation, average calcium intake is less than the recommended intake as early as childhood (Weaver, 1998).

Calcium chloride is a good source of calcium and a good fortifying agent. It is one of the 10 compounds demonstrated by FDA as safe and lawful to use as dietary supplement (56 FR at 60691) to supply calcium.

Manufacturers of calcium-fortified foods should also consider the addition of magnesium, riboflavin or even vitamin D. Vitamin D fortification is not a very common practice and studies have shown that calcium absorption is directly affected by vitamin D level in the body. An individual with a deficiency in vitamin D will absorb less calcium than individuals with adequate vitamin D intake.

### **1.9 – Beta-lactoglobulin (BLG)**

A protein of animal origin, BLG is a polypeptide with 150-170 amino acids (species dependent). It is found in milk of many mammals including cows. BLG is stable in solution at the pH of human stomach acid. As pH changes, the conformation of BLG changes, but not enough as to denature it (Jankowski and Sichel, 2003). BLG has a high binding affinity for retinol and certain fatty acids.

Studies have also shown vitamin D binding to BLG. Vitamin D absorbs light at 287 nm, but when bound with BLG, a spectral shift occurs and the amplitude of fluorescence emission at 412 nm increases. The protein alone is shown to have fluorescence emission at 332 nm. Energy transfer is observed from the tryptophanyl residues of the protein. The binding affinity of vitamin D is similar to that of retinoids (Wang et. al, 1997). Thus a complex between BLG and vitamin D may be a stable and soluble means of fortifying aqueous products, such as sport drinks and bottled water with vitamin D (Swaigood et al., 2001).

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CHAPTER 2  
**SENSORY STUDY**

THRESHOLD AND CONSUMER ACCEPTABILITY STUDY OF CALCIUM  
CHLORIDE USING  
DE-IONIZED WATER, FLAVORED WATER, AND SPORT DRINK

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## **2.1 - Abstract**

In the U.S., 90% of women, 60% of men, and over 50% of children consume less than the RDA for calcium. Calcium intake through dairy product consumption is declining as other beverages replace milk. Calcium fortification of water or flavored waters could increase calcium intake without impacting caloric intake. The objectives of this study were to determine the sensory threshold of calcium chloride in water and flavored water, design an acceptable calcium-fortified beverage.

Mineral content was determined for nine commercial bottled and flavored waters by atomic absorption spectrophotometry. Sensory detection thresholds of calcium chloride were determined in deionized water (DI), tap water, berry flavored water, and a sports beverage formulation using a five series ascending forced choice analysis. Ascending calcium chloride concentrations in the fortified drinks were evaluated by 30 panelists in duplicate trials. Ninety-eight consumers evaluated acceptability of de-ionized and flavored water with and without added calcium chloride.

Calcium concentration of the nine commercial products measured varied from 0.3 mg/L to 116 mg/L. Sensory thresholds for calcium chloride in drinks were: flavored water ( $857 \pm 8.9$  mg/L) > sport drinks ( $844 \pm 9.8$  mg/L) > DI- water ( $101 \pm 3$  mg/L) > tap water ( $93.5 \pm 3$  mg/L), respectively. (On the basis of Ca, thresholds were flavored water ( $7.72 \pm 0.08$  mM) > sport drinks ( $7.60 \pm 0.09$  mM) > DI- water ( $0.91 \pm 0.01$  mM) > tap water ( $0.91 \pm 0.01$  mM), respectively.) Consumer acceptability scores were not different for water or flavored water with and without added calcium chloride (70 mg/L and 700 mg/L, or 0.63 and 6.31 mM, respectively) ( $p > 0.05$ ).

In conclusion, Calcium content in flavored drinks or water can be increased with calcium chloride without impacting acceptability

## **2.2 - Introduction**

Demands for sports drinks, flavored drinks, and bottled waters are increasing as more people seek the benefit of these products. Athletes consume sport drinks during or after exercising for hydration and energy and electrolyte replacement. Flavored waters are preferred to regular water by some consumers because of the flavor and sweetness. Bottled water consumption is still increasing because of its convenience. These drinks will be an ideal medium for calcium fortification especially to the consumer seeking to increase the calcium intake without increasing their calorie intake.

As much as fortification is important, taste also matters to consumers. The ability to fortify a product with a highly bioavailable nutrient and at the same time increase the palatability of the product is a main driving force in food product development. Because of the chalky taste associated with calcium carbonate and the bitter and astringent taste associated with calcium chloride, it is important to mask the taste of these compounds with ingredients that might affect the acceptability of the product (Lawless et al., 2003). The objective of this study was to determine the threshold for taste of calcium chloride in water and flavored beverages to see if the calcium content could be increased in new products relative to products currently marketed.

## **2.3 - Materials and Methods**

### **2.3.1 - Deionized water**

Deionized water was obtained by reverse osmosis filtration, followed by ion exchange to achieve 17 meg-ohm resistances. One third of the water volume was boiled off to remove any odor that might interfere with sensory perception and the remaining

two-thirds volume was used in sensory studies. This water is referred to as deodorized di-ionized water. After cooling, the water was kept in 4-L brown glass bottles until ready to use. This water was used for diluting the sport drink and flavored-water concentrates.

### **2.3.2 - Sport drinks**

Monarch Beverages (Columbus, Georgia) provided a sport drink concentrate (Blue Raz Ice). Calcium was not added to the beverage during formulation, although it is usually present in this product at 90 ppm. The concentrate was diluted as, 5 parts di-ionized water to 1 part sport drink concentrate. The concentrate was stored at room temperature until ready to use.

### **2.3.3 - Flavored-water**

A new formulation of flavored water was developed for this study. A recipe from Mother Murphy's (Greensboro NC) was used initially and later modified. The ingredients used were food grade chemicals donated from various commercial sources. This includes sodium benzoate (Velsicol Chemical Corporation, Rosemont IL.), citric acid, potassium citrate (Tate & Lyle, Decatur IL), Splenda (McNeil Nutritional, Fort Washington, PA), Natural raspberry flavor 0.2% WONF (With Other Natural Flavor) (Philadelphia PA), and calcium chloride (Tetra Technologies, Inc, Woodlands, TX).

#### 2.3.4 - Beverage dilution

The final formulation of the flavored water concentrate is as follows:

**Table 2.1**  
**Flavored water formulation**

| <b><i>Ingredients</i></b> | <b><i>Formulation</i></b> |
|---------------------------|---------------------------|
| <b>Water</b>              | <b>979.7 ml</b>           |
| <b>Benzoate</b>           | <b>1.79 g</b>             |
| <b>Citric acid</b>        | <b>6.29 g</b>             |
| <b>Potassium Citrate</b>  | <b>3.445 g</b>            |
| <b>Flavor concentrate</b> | <b>5 ml</b>               |
| <b>Splenda</b>            | <b>1g</b>                 |

This concentrate was diluted 1 part formulation to 5 parts de-ionized water and appropriate concentrations of calcium chloride were added prior to use in this study.

The Institutional Review Board (IRB) of North Carolina State University approved this study. All participants in this research voluntarily agreed to participate and informed consent forms were read and signed by each participant. Subjects were recruited through flyers and emails sent through the NCSU Department of Food Science.

### **2.4- Experimental Design**

#### **2.4.1- Threshold Study**

Thirty subjects were used for the threshold study. The participants volunteered to attend all the sessions and they were compensated for their time at the conclusion of the study. Informed consent was given. Threshold study design was to determine the sensitivity of  $\text{CaCl}_2$  in tap water, deionized water; a new formulated flavored water, and sport drink. The study was conducted in eight sessions.

In the threshold study design, panelists were given a tray of samples, with 3 samples in a row. Two samples were the same and one sample was different. The different sample was the fortified sample and the two other samples were blanks. The

objective of this study was to determine which sample is different in each row as the concentration of calcium chloride concentration increases with each row sample.

The samples were identified by random three-digit codes (Table 2); the highlighted codes represent the odd sample that contained  $\text{CaCl}_2$ . If the odd sample could not be determined after tasting all samples in a row, panelists were required to guess which sample was different before tasting the next row of samples. Panelists were asked to rinse their mouth with deionized water and eat a cracker (if desired) between samples to prevent carry-over effects. A spit-cup was also provided to the panelists, should they need to spit out the sample

**Table 2.2**

**A typical 5-row sample tray with 3 samples per row**

|       |     |     |     |                                                                               |
|-------|-----|-----|-----|-------------------------------------------------------------------------------|
| Row 1 | 113 | 234 | 003 | <div>Lowest Concentration</div> <div>↓</div> <div>Highest Concentration</div> |
| Row 2 | 530 | 214 | 375 |                                                                               |
| Row 3 | 467 | 992 | 751 |                                                                               |
| Row 4 | 395 | 309 | 974 |                                                                               |
| Row 5 | 588 | 743 | 608 |                                                                               |

Highlighted codes are the odd sample in random order

Following the format shown in Table 2, the concentration of calcium chloride used in each spiked sample is shown in Table 3 for the four threshold trials in this study.

**Table 2.3**  
**Calcium Chloride concentration**

| <b>CaCl<sub>2</sub><br/>concentration ppm<br/>or mM</b> | <b>Tap<br/>Water</b> | <b>De-ionized<br/>Water</b> | <b>Flavored<br/>Water</b> | <b>Sport<br/>Drink</b> |
|---------------------------------------------------------|----------------------|-----------------------------|---------------------------|------------------------|
| Lowest                                                  | 37.5<br>0.34         | 44<br>0.40                  | 176<br>1.59               | 200<br>1.80            |
|                                                         | 75<br>0.68           | 88<br>0.79                  | 352<br>3.17               | 400<br>3.60            |
|                                                         | 150<br>1.35          | 176<br>1.59                 | 704<br>6.34               | 800<br>7.21            |
|                                                         | 300<br>2.70          | 352<br>3.17                 | 1408<br>12.69             | 1600<br>14.42          |
| Highest (ppm)                                           | 600<br>5.41          | 704<br>6.34                 | 2816<br>25.37             | 3200<br>28.83          |

A preliminary test was conducted using twelve subjects to determine an acceptable concentration of calcium chloride before the threshold test. These tests were performed in duplicate trials and the concentration range was a geometric progression. The threshold test for each medium was also performed in duplicate trials. Data were collected and geometric means were used to calculate the threshold value.

#### **2.4.2- Consumer acceptance study**

This study was conducted with ninety-eight people; subjects were recruited through flyers, word of mouth, and emails at the North Carolina State University Department of Food Science. Questionnaires and consent forms were given to each subject upon arriving at the sensory booth. No training session was required and samples were given to subjects in random order. The media used for the acceptance study were berry-flavored water and de-ionized water.

A three-digit code was used to denote each sample. Between each sample panelists were required to rinse with deionized water, and eat a cracker if desired. All samples were served at refrigerator temperature.

The forced choice method used is a non-quantitative test that concludes whether a difference exists between products, but not how it exists. For each medium, the panelists were given 2 samples, one a fortified sample and the other a non-fortified sample.

The objective of this study was to determine the consumer acceptability of each sample based on three criteria:

- Thirst Quenching Ability of the sample
- Overall Flavor liking of the sample
- Overall liking of the sample

Since the berry-flavored water contained Splenda, a sweetener, de-ionized water samples were presented first to the panelists to prevent carry-over effects from the sweetener. Data were organized using Excel™ and the Analysis of Variance procedure of SAS (Cary, NC) was used to analyze the data from each trial.

#### **2.4.3- Atomic Absorption Spectrophotometry (AAS)**

Ten commercial products were tested to determine their calcium content. The drinks (bottled water & flavored/sport drinks) were purchased at local grocery stores. The calcium content of these drinks was measured using an atomic absorption spectrophotometer (Perkin Elmer model 3100). The objective of the atomic absorption measurements for this study was to compare calcium concentration in these products to our threshold data for calcium chloride.

## **2.5- Results**

Calcium chloride threshold was different in each medium (Table 2.4). The intensity of calcium chloride was higher in water than in flavored products. Panelists categorized calcium chloride flavor as bitter, astringent, and salty.

**Table 2.4**  
**Threshold data of Calcium Chloride in different media**  
**(mg CaCl<sub>2</sub>/L, mg Ca/L, and mM)**

| <b>Tap water</b>   | <b>De-ionized water</b> | <b>Flavored water</b> | <b>Sport Drink</b> |
|--------------------|-------------------------|-----------------------|--------------------|
| 93 ± 3 mg/L        | 101 ± 3 mg/L            | 857 ± 8.9 mg/L        | 844 ± 9.8 mg/L     |
| 33.8 ± 1.1 mg Ca/L | 36.5 ± 1.1 mg Ca/L      | 309 ± 3.2 mg Ca/L     | 305 ± 3.5 mg Ca/L  |
| 0.84 ± 0.01 mM     | 0.91 ± 0.01 mM          | 7.72 ± 0.08 mM        | 7.60 ± 0.09 mM     |

Calcium content of commercial products tested using AAS showed low calcium concentration relative to the CaCl<sub>2</sub> threshold data. The results are tabulated in Table 2.5.

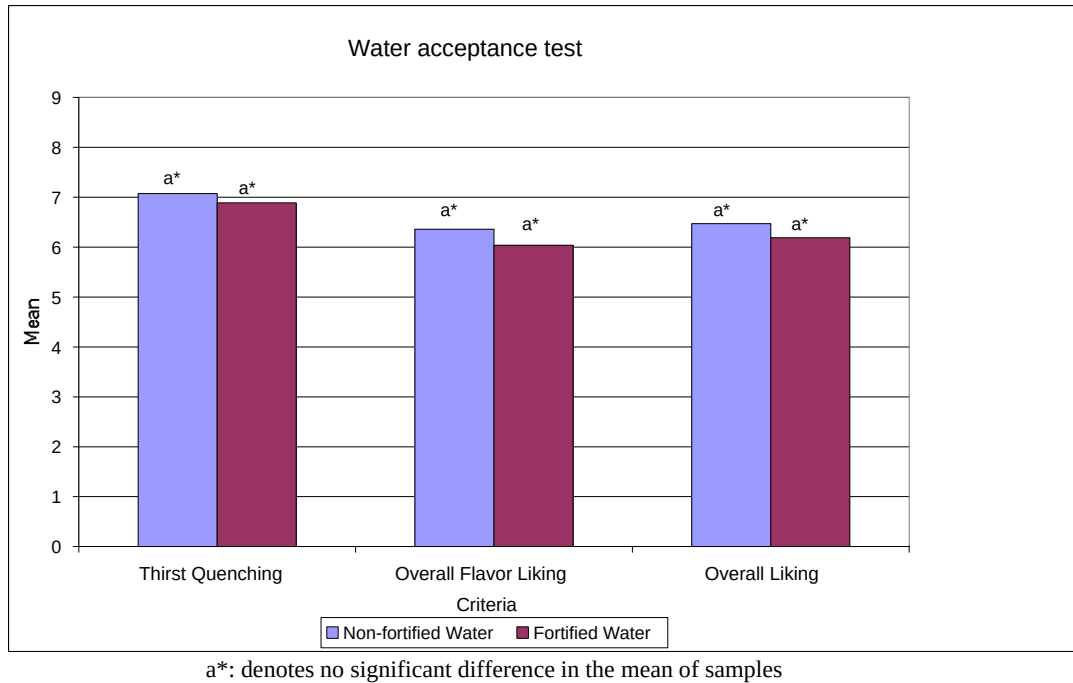
**Table 2.5**  
**Comparison of commercial product for calcium with AAS**

| <b>BOTTLED WATER</b>          | <b>Calcium concentration</b> |           |
|-------------------------------|------------------------------|-----------|
|                               | <b>(mg/L)</b>                | <b>mM</b> |
| Aquafina                      | 0                            | 0         |
| Desani                        | 0.03                         | 0.001     |
| Evian                         | 87.5                         | 2.18      |
| Fresh Samantha Spring Water   | 3.13                         | 0.078     |
| Glaceau Smart Water           | 4.77                         | 0.119     |
| Le Bleu                       | 0                            | 0.000     |
| 365 Whole Foods Mineral Water | 116                          | 2.89      |
| <b>FLAVORED DRINKS</b>        |                              |           |
| Gatorade                      | 8.40                         | 0.210     |
| Glaceau Wellness Water        | 8.92                         | 0.223     |
| Powerade                      | 16.8                         | 0.419     |
| <b>MILK PRODUCTS</b>          |                              |           |
| Whole                         | 1030                         | 25.7      |
| Reduced Fat                   | 1070                         | 26.7      |
| Skim                          | 1140                         | 28.4      |

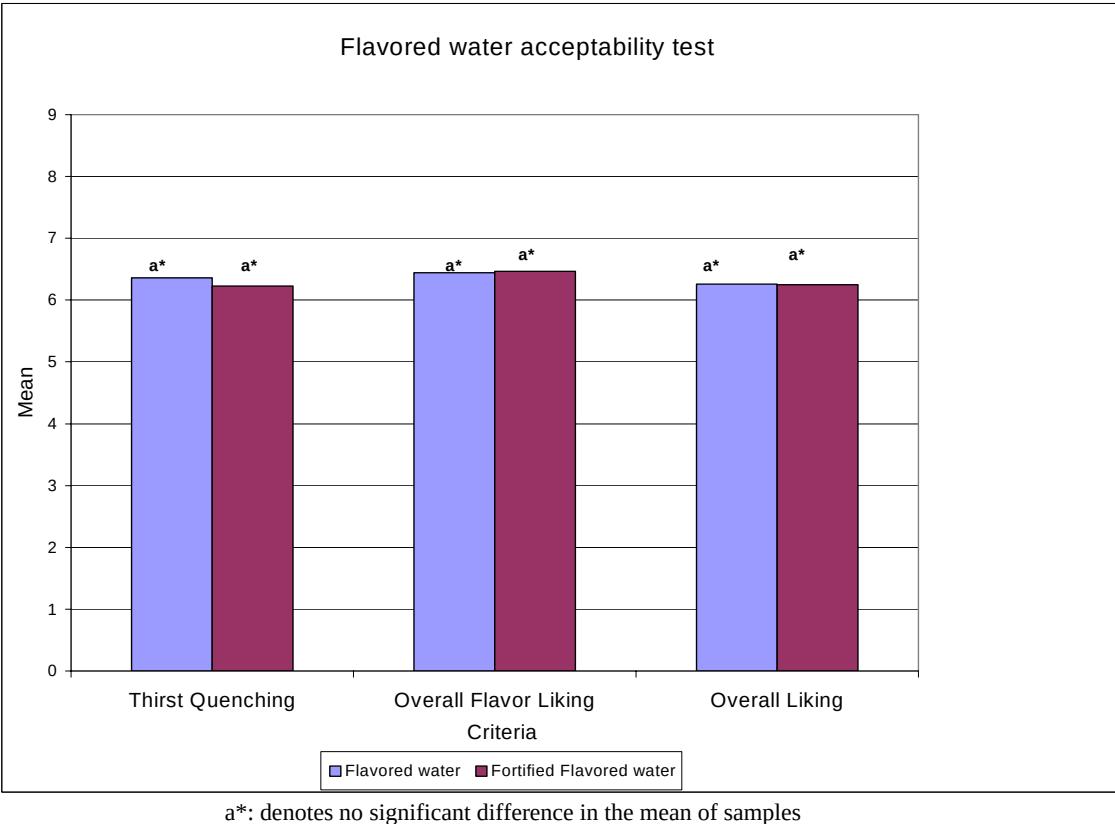
The threshold of calcium chloride in flavored-water and regular water was 857 mg/L and 101 mg/L respectively, but for the consumer acceptance test, 700 mg/L and 70 mg/L of calcium chloride were used, respectively. This concentration of calcium chloride was chosen because at the average threshold concentration, approximately half of the panelists would easily detect it. This threshold is sometimes called recognition threshold, defined as the concentration at which stimuli is detected and recognized. But by using a lower concentration of calcium chloride, absolute threshold can be detected. Absolute threshold is defined as the lowest concentration capable of producing stimuli (Lawless et al. 1999).

Data from the consumer acceptance study are shown in Figures 2.1 and 2.2. The mean of the overall flavor liking, overall thirst quenching potential and overall liking did not differ between the the 70 mg/L  $\text{CaCl}_2$ -fortified water and non-fortified water (Figure 2.1). Likewise, 700 mg/L  $\text{CaCl}_2$ -fortified flavored water and non-fortified flavored water did not differ (Figure 2.2). The acceptance for both fortified flavored water and non-fortified flavored water was the same.

**Figure 2.1: Water acceptability test with all 3 criteria on a 9-point hedonic scale**



**Figure 2.2: Flavored-water acceptability test with all 3 criteria on a 9-point hedonic scale**



## **2.6 Discussion**

The threshold of calcium chloride was lower in water and de-ionized water than in flavored-water and sport drinks. Flavored-water and sport drinks contained a significant amount of sweetener and acid, and these compounds have been shown to mask some calcium chloride attributes like saltiness, bitterness, and astringency (Lawless et al. 2003). The sweetness and acidity of these drinks increases the threshold for calcium chloride allowing use of higher concentrations of calcium chloride. Compared to the current concentration of calcium chloride in the sport drink used in this study (Blue Raz Ice, Monarch beverages), the threshold data shows that at least 10 fold more calcium chloride could be added to this drink before being detected by most people.

With the exception of the mineral water and milk, the calcium levels in these commercial products were low indicating that more calcium salt could be added to these products if fortification were desired. The concentration of calcium chloride added had no impact on the acceptability of the water and flavored water products tested by consumers. Some of the characteristics of calcium chloride are bitterness, sourness and sweetness (Lawless et al., 2003), but these characteristics were undetectable at the  $\text{CaCl}_2$  concentrations used in this experiment.

The flavored water used in the consumer panel contained Splenda, a sweetener. This sweetener could have resulted in a cross-adaptation, whereby the adaptation to the sweet compound causes a decrease in the adaptation of the calcium chloride compound. The sweetener may have also acted as a suppressant since it is known to suppress bitterness. The concentration of calcium chloride tested in water was low and

nutritionally insignificant compared to the concentration of calcium chloride in flavored water and sport drink.

## **2.6 - Conclusion**

The sweetness and acidity of the flavored-water and sport drink help to increase the threshold for calcium chloride. In water, a low concentration of calcium chloride was detectable. The threshold concentration of calcium chloride in water is higher than most of the commercially available products tested using AAS, even though this concentration is nutritionally insignificant.

Because both the fortified and the non-fortified flavored water and sport drinks were equally acceptable to panelists, consuming the fortified drinks on a regular basis might have a health benefit associated with the increased calcium intake. This includes increasing the calcium consumption in the diet without increasing calorie intake, especially with flavored water, which has low calorie content. An adult could obtain more than 20% of the Dietary Reference Intake (FNB, 1997) or Daily Value for calcium by consuming one liter per day of flavored water fortified with 700 mg/L of  $\text{CaCl}_2$ . However fortifying bottled water to the threshold concentration (90-100 mg  $\text{CaCl}_2$ /L) would provide only about 3% of the Daily Value for calcium (1000 mg Ca/day). Additional studies are needed to see if higher levels of  $\text{CaCl}_2$  in bottled water would be acceptable to consumers, even though the taste is perceptible. The successful marketing of Evian and Whole Foods Mineral Water suggests that a perceptible Ca flavor may be acceptable to consumers.

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**CHAPTER 3**  
**BLG-VITAMIN D BINDING BY FREEZE-DRYING**

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### **3.1 - Introduction**

Beta-lactoglobulin (BLG) is a member of the lipocalin family of proteins, which are known for transporting various physiologically important molecules. BLG tightly binds cis-retinol (vitamin A) (Jankowski et al, 2002) and related molecules with a possible transport function. The binding affinity of retinol, fatty acid and other compounds to BLG has been reported by Wang (1997). Binding of inhibitory fatty acids to BLG enhances the pregastric esterase activity (Pérez et. al, 1995). Substitution of Trp19 of BLG can lead to fluorescence changes when retinol binds to BLG and similar changes have been observed with vitamin D binding (Dufour et. al, 1994 and Wang et. al, 1997). Fluorescence emission spectra of BLG were recorded at 287 nm and after binding with vitamin D<sub>2</sub> and vitamin D<sub>3</sub>, fluorescence emission was increased between 300 nm-360 nm for vitamin D<sub>2</sub> and at 412 nm with vitamin D<sub>3</sub>

Freeze-drying (lyophilization) is the process of removing water from a product by sublimation. Water molecules in a solid phase material are converted to free water molecules in the vapor phase. The water molecules are removed from the solid by low atmospheric pressure during the freeze drying process. The first step as described above is sublimation, and then a secondary drying removes non-frozen bound water.

The purpose of the experiment was to freeze-dry a complex between BLG and Vitamin D that may be a stable and soluble means of fortifying aqueous products, such as sport drinks and bottled water with vitamin D (Swaigood et al., 2001). Measuring the bioavailability and nutritional interactions of CaCl<sub>2</sub> and vitamin D in non-fat beverages required that we create a model Ca/vitamin D-fortified water that could meet calcium

requirements of rats and study it as a model system for calcium/vitamin D-fortified bottled water for human consumption.

### **3.2 - Materials and Methods**

Davisco Foods International, Inc. (Le Sueur, MN) supplied pure BLG. Vitamin D<sub>3</sub> was purchased from Sigma Chemical Co. (St. Louis, MO), while vitamin D<sub>2</sub> was purchased from Roche Vitamins Inc. (Nutley, NJ). Two grams of BLG were dissolved in 30 ml deionized-water and mixed with 0.25 g of vitamin D<sub>3</sub>. The mixture was stirred overnight on a magnetic stirring plate using a low speed of 2 to prevent foaming. If the mixture were stirred or vortexed at high speed, it could denature BLG, reducing the effectiveness of BLG-vitamin binding. All work with vitamin D was conducted in the dim yellow light with containers wrapped in aluminum foil to protect the BLG complex from direct light.

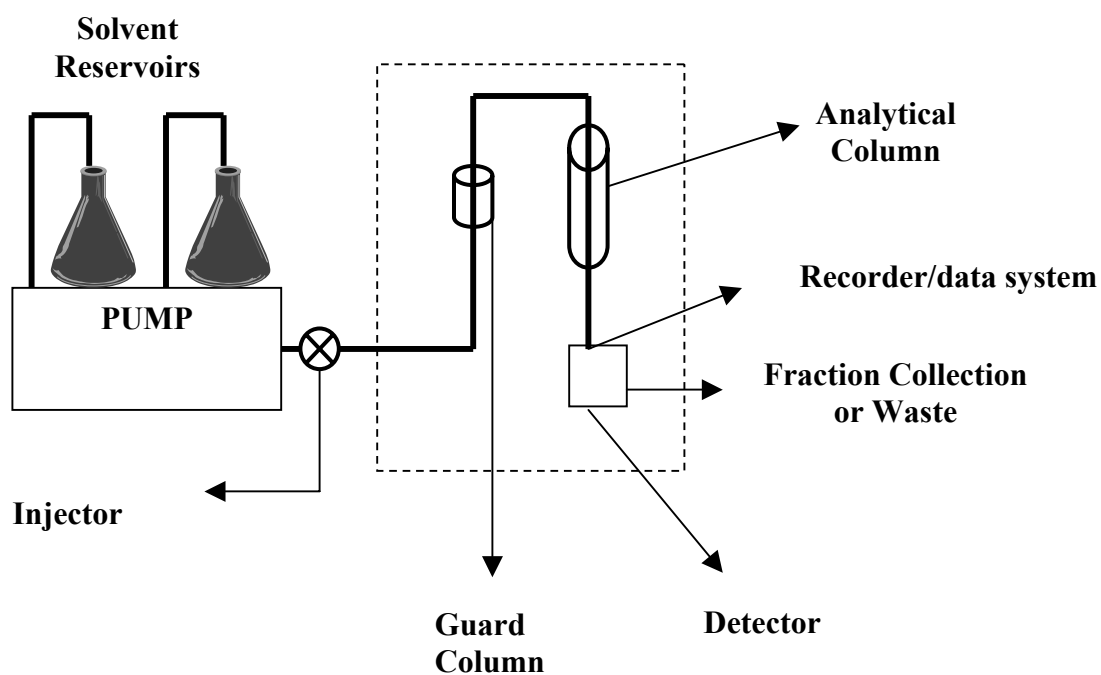
The freeze-drying method consists of freezing the protein solution and subsequently drying the solid under vacuum. This is a two-phase method that first removes the water through sublimation and then removes the non-frozen bound water. Equal aliquots of the mixed BLG-vitamin D were placed in the freeze-drying bottles (Labconco). The bottles were rotated in a methanol/dry ice mixture until the solution was completely frozen. After freeze-drying, the bottles were tightly wrapped in foil to prevent direct light from degrading the vitamin D.

A Freeze Dryer 5 (Labconco Co., Kansas City, MO.) was used for the sublimation phase. The condenser was turned on and left running until the temperature of the condenser reached 0°C. The vacuum pump was turned on and the bottles were connected

to the outlets. For proper sealing, the unused outlets were sealed with rubber stoppers. The valve of each connected outlet was moved from vent to vacuum. The drying phase requires 24 hr or longer, depending on the amount of liquid in each bottle. After drying, the valve between each bottle outlet and the drier was turned back to vent and bottles were detached from the outlets. The recovered powder was whitish in color and fluffy. The powder was transferred to a brown bottle and kept in dark conditions until use.

**Figure 3.1**

**HPLC analysis of vitamin D in BLG-vitamin D powder**



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

HPLC analysis used a Waters 510 pump, a gradient controller, UVIS linear detector, and a manual loading injector (Waters Associates, Milford MA). The mobile phase was acetonitrile: ethyl acetate: chloroform (88:8:4 v/v/v) at a flow rate of 1 ml/min. A reverse

phase, 4.6 x 250 mm Vydac TP201 C18, 5 µm column with a guard column (Vydac, Hisperia CA) was used. The flow program used for column conditioning and equilibration is shown in Table 3.1.

**Table 3.1 - Flow program used for column conditioning and equilibration.**

| 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.

### **3.2.1 - Standard Curve and Recovery Study of Vitamin D from BLG**

#### **Standard Curve**

Methanol, 100 ml, was added to 0.02 g of Vitamin D<sub>2</sub> and D<sub>3</sub> for a final concentration of 200 ug/ml. A 30-ml stock of Vitamin D<sub>2</sub> and D<sub>3</sub> was made and stored at 4° C until use. To determine the concentration of vitamin D<sub>2</sub> and D<sub>3</sub> in the BLG powder, a 1:10,000 dilution was made and tested on a Genesys 2 spectrophotometer (Spectronic, Thermo Electron Scientific Instruments Co. Madison WI).

The calculation used to determine concentration is as follows:

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

C= concentration

A= absorbance @ 264nm

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

B= length = 1cm

The concentration of vitamin D<sub>2</sub> and D<sub>3</sub> in these working standards was collected as follows.

**Table 3.2 Vitamin D concentrations in standards.**

| Samples | Volume of D <sub>2</sub> Vitamin D <sub>2</sub> working solution (1.52µg/ml, 90µl = .14µg) | D <sub>2</sub> Vitamin D <sub>2</sub> concentration (µg/ml) | Volume of D <sub>3</sub> Vitamin D <sub>3</sub> working solution (2.03µg/ml, 24µl = .05µg) | D <sub>3</sub> Vitamin D <sub>3</sub> concentration (µg/ml) |
|---------|--------------------------------------------------------------------------------------------|-------------------------------------------------------------|--------------------------------------------------------------------------------------------|-------------------------------------------------------------|
| 1       | 90                                                                                         | 0.14                                                        | 24                                                                                         | 0.05                                                        |
| 2       | 90                                                                                         | 0.14                                                        | 48                                                                                         | 0.10                                                        |
| 3       | 90                                                                                         | 0.14                                                        | 96                                                                                         | 0.20                                                        |
| 4       | 90                                                                                         | 0.14                                                        | 192                                                                                        | 0.40                                                        |

For developing a HPLC standard curve, samples of vitamin D<sub>3</sub> were added to 4 separate brown vials and dried with N<sub>2</sub> gas (N-Evap N<sub>2</sub> dryer, Mayer Analytical Co.) Upon drying, vitamin D<sub>2</sub> was added to respective vials and mixed thoroughly. 50 µl of methanol were added to re-dissolve the vitamin D (Table 3.2). Fifty-µl of each sample was injected for HPLC analysis.

### **3.2.2 - Assay of vitamin D in water (Murphy 2001)**

Because the vitamin D<sub>3</sub> was in water, no saponification step was needed. Medium containing fat must be saponified to break down or degrade fat by treating the fat with alkali like KOH.

The assay was performed in duplicate. 5mg of BLG/ vit. D mix + 100 ml of vitamin D (2ug/ml) + 15 ml deionized water were mixed together, for a total of 15.1ml sample. The 15.1-ml samples were transferred to 125-ml actinic separatory funnel and 15

ml of distilled water, 5 ml of absolute ethanol, and 45 ml of hexane were added. Samples in the separatory funnel were shaken for 1 min and left undisturbed for 4 min. The aqueous layer was then transferred to an Erlenmeyer flask and the organic layer was transferred to a 250-ml actinic separatory flask. The aqueous layer was then returned back to the 125-ml separatory flask and 45 ml of hexane were added. The flask was shaken for 1 min and left undisturbed for 4 min. The last two steps were repeated, keeping the organic layer by transferring to the 250-ml actinic separatory flask. After repeating the last two steps once, the aqueous layer was discarded. Fifty ml of 5% KOH was added to the 250-ml actinic separatory flask containing the organic layer and it was shaken for 1 min and left undisturbed for 4 min. The aqueous layer was then discarded. Fifty ml of distilled water were added to the 250-ml actinic separatory flask; the flask was shaken for 1 min and left undisturbed for 4 min. The aqueous layer was then discarded. Fifty ml of 55% ethanol: water was added to the 250 ml actinic separatory flask; the flask and its content were shaken for 1 min and left undisturbed for 10 min. The aqueous layer was then discarded. The organic layer was transferred to a 250-ml round bottom flask and evaporated to dryness at 50° C using a Rotavapor R-124 (BUCHI Analytical Instruments). The temperature of the water bath was kept below 50° C to prevent over-heating and destruction of vitamin D. Upon dryness, 5 ml of hexane were immediately added to the flask containing residues of vitamin D, swirled and transferred to centrifuge tubes. This rinse was repeated twice, once with 3 ml of hexane and then 1 ml of hexane. The washes were collected in centrifuge tubes. The solvent in the centrifuge containing the vitamin D residues was removed under a stream of N<sub>2</sub> and the residue was then re-dissolved in 500 µl methanol. This solvent/vitamin D was then

filtered through a 0.45- $\mu$ m syringe filter into a small 2-ml amber vial. N<sub>2</sub> gas was applied quickly to the vial to remove any oxygen present in the vial. Samples were kept at -20° C until use.

### **3.2.3 - Recovery of vitamin D from BLG powder**

Fifty  $\mu$ l of the extracted sample was injected into the HPLC. Vitamin D standards were injected first before injecting the unknown samples collected from recovery steps. Area under curve (AUC) was recorded for vitamin D<sub>2</sub> and D<sub>3</sub> for each sample. The retention time for both vitamins was also recorded.

## **3.3 - Results**

**Figure 3.2**

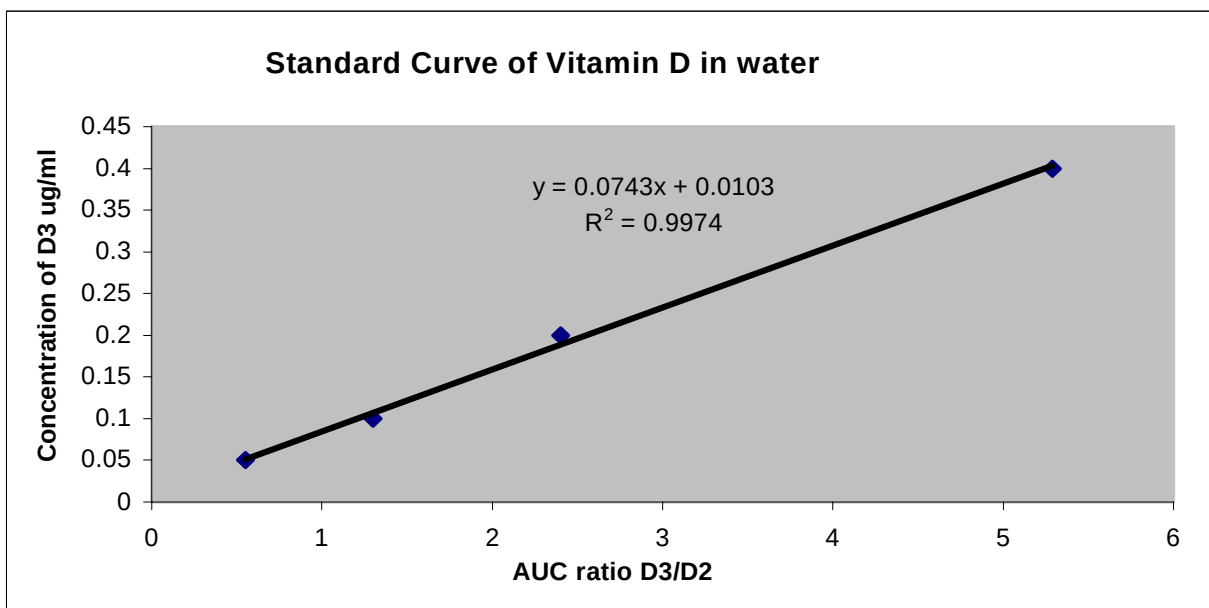


Figure 3.2 shows the standard curve of vitamin D in water. The retention time for vitamin D<sub>2</sub> and D<sub>3</sub> is 11.016 min and 12.916 min.

**Table 3.3**  
**AUC of vitamin D<sub>2</sub>**

| Sample                                     | AUC in standard curve | AUC in recovery study | Recovery |
|--------------------------------------------|-----------------------|-----------------------|----------|
| #1                                         | 563689                | 518650                | .9200    |
| #2                                         | 655858                | 486864                | .7423    |
| Mean = .8312 and Standard Deviation = .125 |                       |                       |          |

**Table 3.4**  
**AUC of vitamin D<sub>3</sub>**

| Sample                                     | AUC in standard curve | AUC in recovery study | Recovery |
|--------------------------------------------|-----------------------|-----------------------|----------|
| #1                                         | 1749526               | 1468916               | .8396    |
| #2                                         | 864385                | 467453                | .5408    |
| Mean = .6902 and Standard Deviation = .211 |                       |                       |          |

An extraction from four random samples was used to determine the concentration of vitamin D in the BLG complex. An average concentration of vitamin D in the BLG-vitamin D complex was 0.1774 µg vitamin D<sub>3</sub> /mg BLG. Since the vitamin D-BLG complex was designed as a model system to create a model Ca/vitamin D-fortified water that could meet calcium and vitamin D requirements of rats. The amount of BLG complex added into rat's water was 162 mg BLG complex/2 Liter water to achieve a final concentration of 14.4 µg vitamin D /L (chapter 4, table 4.1).

### **3.4 - Discussion**

Vitamin D has been shown to bind with beta-lactoglobulin (BLG) and is known to fluoresce at 287 nm excitation. The change in energy transfer is due to vitamin D binding to BLG, which results in the excitation of the tryptophanyl residues with

maximum excitation of 412 nm. If vitamin D were bound, fluorescence at this wavelength would be expected (Wang et. al, 1997).

The extraction method used in this experiment (Murphy 2001) had been developed for milk, which is known to contain a significant amount of fat. Water, on the other hand, contains no fat that could aid in vitamin D solubility. The extraction method and HPLC program used has been proven successful for vitamin D recovery from milk. Low recovery of vitamin D<sub>2</sub> and D<sub>3</sub> was observed, compared to other studies in which milk was the matrix containing vitamin D (Faulkner et.al, 2000 and Murphy et. al 2001). Other peaks representing unknown compounds were observed on the HPLC chromatograph before elution of vitamin D<sub>2</sub> and D<sub>3</sub>; such peaks were unexpected because deionized water was used as the vitamin D-BLG solvent. Such peaks might be expected in milk samples, which might contain other compounds. The protocol used by Murphy (2001) contained a saponification method, which was not used in our study because water contains no fat. The saponification method was used to degrade any neutral fat present in the medium.

The objective of this study was to develop a model Ca/vitamin D fortifier for use in bottled water and beverages like sport drinks or soft drinks. The concentration of calcium used in our model beverage was based upon feed and water intake by five 13-week old Wistar rats measured in a preliminary experiment in our laboratory. The Ca/vitamin D fortified solutions were to be tested for bioavailability in a rat model system. In rats' diet, the normal calcium concentration is 5 mg/g and the average ratio of food to water intake in the preliminary study was 0.576 (Liu et al., 2003). Therefore, 2.88 g Ca/L and 14.4 g vitamin D/L in drinking water were estimated to meet the nutrient

requirements for rats. The ratio of vitamin D and BLG used to formulate the vitamin-protein complex was based on previous research that shows a 1:1 protein (BLG)/ vitamin molar ratio in formulating a freeze-dried BLG/vitamin D complex (Wang et al., 1999.)

### **3.5 - Conclusion**

In future work, a better analytical method should be designed for extraction of vitamin D from aqueous solutions, because the poor recovery was a limitation with the protocol used. There is not any published work performed with vitamin D extraction from water, but as fortification continues to increase and demand for fortified food increases, more research in this area is expected. As noted, the vitamin D is insoluble in water, but vitamin D was bound to BLG which is soluble in water. Shelf stability testing was not performed to determine how stable the BLG-vitamin D is in water.

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**CHAPTER 4**  
**THE BIOAVAILABILITY OF VITAMIN D AND CALCIUM IN RATS**

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#### **4.1 - Abstract**

The nutritional significance of calcium in bottled water is often questioned. The content in a serving for many brands of spring water is usually less than 2% of the Daily Value. Mineral and fortified water can have nutritionally significant quantity. Our objective was to determine the bioavailability of calcium from calcium chloride-fortified drinking water in vitamin D-deficient rats. Vitamin D-deficient diets had either low (0.2%) or normal (0.5%) calcium content. After a 2-wk depletion phase, drinking water fortified with calcium chloride calculated to provide 0, 0.25, 0.5 or 1.0 times the usual calcium intake (estimated at 2.88 g/L) from diet was provided. Additional groups also had water fortified at 2 x usual dietary intake, but did not complete the study because of low feed and water intake. Some groups also had a water-soluble form of vitamin D added to the water. However, the final serum vitamin D level indicated that the vitamin D in water was poorly available to the rats. Results showed that groups fed low dietary calcium and not supplemented with calcium in water had lower bone weight, bone ash weight, and bone ash calcium percentage, than did the rats supplemented with calcium in water. The deficient rats with no calcium and low vitamin D and those with highest Ca (2.88 g/L) in water all had lower final body weight than the control group (Ca and vitamin D in diet), and the groups with moderate water Ca (1.44 g/L). Body weight was correlated with food intake. For groups with 0.5% dietary Ca, bone weight, size, Ca, or breaking strength was not related to the Ca or vitamin D intake from water. Fortifying drinking water with calcium chloride significantly reduced the effect of low dietary Ca intake, but had little effect when dietary calcium was adequate.

## **4.2 - Introduction**

Vitamin D is important for homeostatic control of calcium and phosphorus. Vitamin D hormone helps in increasing serum calcium which can be used for skeletal mineralization if dietary calcium intake is adequate (Holick, 1999). With low dietary calcium, activation of vitamin D will remove calcium phosphate from bone in order to maintain normal serum calcium. The objective of this study was to investigate the interaction of calcium chloride and vitamin D in drinking water for rats using bone mineralization as an indicator of calcium status. The results could potentially be applied in formulation of bottled water fortified with calcium and vitamin D. Calcium chloride, as a highly soluble salt, is appropriate for this purpose. Adding Vitamin D to water was possible by use of a water-soluble protein complex between vitamin D and BLG (Swaigood et al., 2001). Vitamin D deficiency in rats fed a low calcium diet has been previously used as a model system for calcium and vitamin D bioavailability (Iwamoto et. al., 2004). The availability of a water-soluble form of vitamin D permitted this experimental design, in which vitamin D was not formulated into the diets of the rats, but was added to the water. Calcium chloride was also added to the water consumed by the separate groups of rats at concentrations designed to map the response surface for the calcium-vitamin D interaction. Two different concentrations of calcium were formulated into the diet to determine if calcium and vitamin D in drinking water affected rat growth and bone deposition at both adequate and low calcium intake.

## **4.3 - Materials and Methods**

Male, 21-day old, Wistar, rats were obtained from Charles River Laboratory (Raleigh, NC). Throughout the experiment the rats were kept in a 27° C room under

incandescent lighting with a 12-h light/dark cycle. The rats were individually housed in wire cages and allowed ad libitum access to food (Dyets® Inc., Bethlehem PA). All rats were fed vitamin D-free, modified AIN-93G diet with normal (5.0 g Ca/kg) or low (2.0 g Ca/kg) calcium level depending on experimental trial. Upon arriving, 5 rats were sacrificed immediately for the baseline data and samples collected from these rats were stored at -20°C until analyses. After a 3-week depletion phase on the vitamin D-deficient diet, the rats' serum 25-OH D<sub>3</sub> was undetectable by radioimmunoassay (DiaSorin, Stillwater, MN) and 10 rats were sacrificed. The serum and femur bones were collected for analysis. The remaining rats were randomly distributed in groups of five into different repletion treatments. For 5 weeks they were given water fortified with different concentrations of CaCl<sub>2</sub> and BLG- vitamin D complex as a source of vitamin D. Because the rats' serum 25-OH D<sub>3</sub> was depleted after 3 weeks, rats fed a vitamin D fortified water should increase their serum 25-OH D<sub>3</sub> at the end of the 5-wk repletion phase.

An incomplete block design was used to group the rats into the following subgroups for drinking water supplementation. The concentrations represent multiples of dietary requirements, equivalent to 2.88 g Ca/L and 14.4 µg vitamin D<sub>3</sub>/L water. This is the estimated daily requirement (EDR) for calcium and vitamin D respectively, based upon feed and water intake by Wistar rats of similar ages in a preliminary experiment in our laboratory.

Table 4.1

**An incomplete block design for rats grouping**

| <b>Vit.D<br/>Ca</b> | <b>0x EDR</b>  | <b>.25x EDR</b> | <b>.50X EDR</b> | <b>1.0x EDR</b> | <b>2.0x EDR</b> |
|---------------------|----------------|-----------------|-----------------|-----------------|-----------------|
| <b>0x EDR</b>       | <b>Group A</b> |                 | <b>Group B</b>  |                 | <b>Group C</b>  |
| <b>.25x EDR</b>     |                | <b>Group D</b>  |                 | <b>Group E</b>  |                 |
| <b>.50x EDR</b>     | <b>Group F</b> |                 | <b>Group G</b>  |                 | <b>Group H</b>  |
| <b>1.0x EDR</b>     |                | <b>Group I</b>  |                 | <b>Group J</b>  |                 |
| <b>2.0x EDR</b>     | <b>Group K</b> |                 | <b>Group L</b>  |                 | <b>Group M</b>  |

*Estimated Daily Requirement (EDR) for vitamin D = 14.4µg/L*

*Estimated Daily Requirement (EDR) for calcium = 2.88g/L*

Five rats were assigned to each group. Positive control group rats (Group Q) were given regular water and regular AIN-93G purified diet which contains 0.25 g/kg vitamin D in the vitamin mix, or 25 µg cholecalciferol /kg diet (Reeves et al., 1993) and 357 g/kg calcium carbonate (used at 35 g/kg diet) in the mineral mix in the formulation of the control diet. Diet A (Normal calcium diet) contained no calcium or vitamin D in the mineral mix or vitamin mix respectively, but 12.5 g/kg of calcium carbonate (40.04% Ca) in the diet formulation.

Diet B (Low calcium diet) also contained no calcium or vitamin D in the mineral mix and vitamin mix respectively, but 5% calcium carbonate (40.04% Ca) was included. Rats in all groups (except group Q) received fresh fortified water weekly in glass water bottles as the only source of vitamin D. Food consumption and rat weight gain was measured and collected weekly. Rats were sacrificed with a ketamine (650mg/100g body weight) + xylazine (140mg/100g body weight) anesthetic to achieve unconsciousness, followed by exsanguination. Blood was removed by cardiac puncture using 10-ml

syringes and 20 GA needles. The left femur was removed for bone ash content and calcium content. The right femur was used for bone strength testing. Statistical analysis of experimental data included the Duncan Waller K-ratio t-test and a correlation test using SAS (Cary, NC).

#### **4.3.1 - Bone Assay for Calcium Analysis**

All work was performed in a fume hood and all tools used including test-tubes, glassware, and crucibles were acid-washed. The left femur was cleaned by removing tissues adhering to the bone. The bones were individually labeled and soaked overnight in a test-tube containing 100% ethanol. The solvent was placed in hazardous waste containers for disposal. The bones were then individually soaked in chloroform overnight. Afterward, the solvent was properly disposed of as hazardous waste. The bones were left to air-dry for about 5 min.

Ceramic crucibles were acid-washed in 1 N HCl and baked in a muffle furnace at approximately 700° C for 2-3 hrs and let cool in desiccators until ready for weighing. Each crucible weight was recorded. Each bone was placed in a crucible, weighed and labeled. This was the wet weight. The bones were then baked in the crucibles at 100° C oven for 8-12 hours. The bones were then transferred to the desiccators to cool. After cooling, the bones were weighed again with the crucible. This was the dry weight. After weighing, the bones were placed in a furnace and ashed at 649° C for 24 hours, and cooled for up to 8 hr. After cooling in desiccators, crucibles were reweighed. This was the ashed weight.

The ashed bones were carefully transferred to a vial and dissolved in 10 ml of 3 N HCl and then diluted 1:1000 using 0.5% lanthanum in 0.1 N HCl. Calcium content in ashed bones was measured using atomic absorption spectrophotometry (Perkin Elmer Model 3100, Norwalk, CT).

#### **4.3.2 - Serum analysis for calcium**

Blood collected was transferred into a 4-ml Vacutainer tube, coated with clot activator. The blood was left to clot for 30 min and later centrifuged at 760 x g for 15 min to separate the serum from the cells. Serum collected was stored in amber vials at -20°C. An atomic absorption spectrophotometer (Perkin Elmer Model 3100, Norwalk, CT) was used to determine calcium content in the serum. The serum was diluted 1:50 using 0.5% lanthanum in 0.1 N HCl and calcium content was determined.

#### **4.3.3 - Analysis for serum 25-OH vitamin D<sub>3</sub>**

25-OH vitamin D <sup>125</sup>I RIA Kit (DiaSorin Co. Stillwater, MN) was used for serum 25-OH vitamin D analysis. This was the first kit approved by Food and Drug Administration (FDA) for assessing 25(OH) D status and it has been proven to work effectively. According to a study comparing two RIA methods for determining circulating 25(OH)D, the <sup>125</sup>I RIA Kit was proven superior (Hollis, 2000).

The collected frozen serum prepared as described above was used for this analysis. The procedure used in this analysis was as directed by the manufacturers' product inserts. 12 x 75 mm disposable glass tubes were labeled accordingly for standards, controls and samples. 500 µl of acetonitrile was added to each tube and 50 µl

of standard, control or sample was carefully and slowly placed by pipetting below the surface of the acetonitrile and vortexed for 10 seconds.

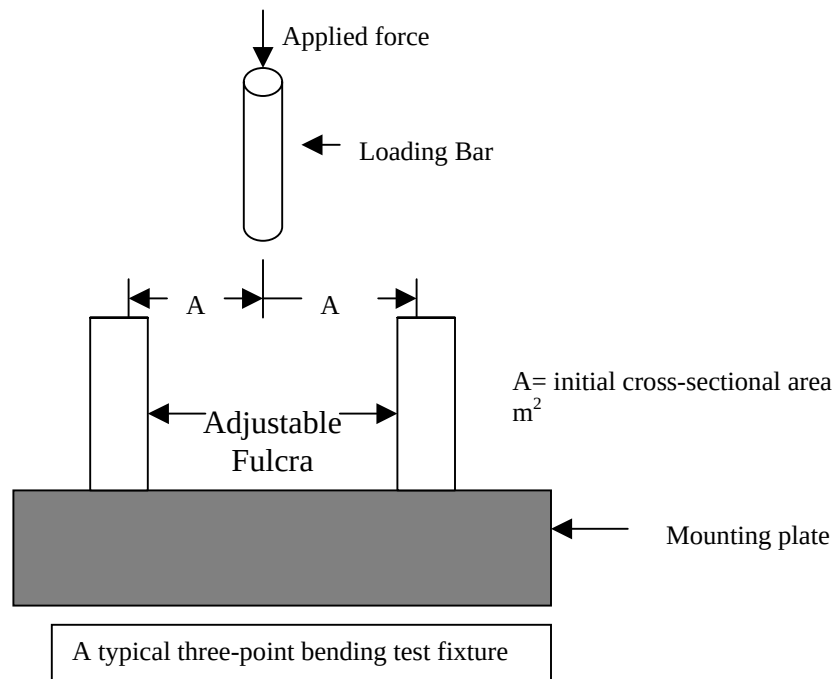
The reagents were then centrifuged at 1200 x g for 10 minutes at 20-25°C. Duplicate 25-µl aliquots from the supernate were pipetted into separate, labeled, 12 x 75 mm tubes. All reagents and samples used were equilibrated to room temperature.

Tubes were labeled as Total count tubes, non-specific binding (NSB), standards, controls, and samples and 25 µl of standards, controls, and samples were added to each appropriate tube. Fifty µl of <sup>125</sup>I 25-OH-D radioactive tracer was added into each tube and 1 ml of NSB/addition buffer was added to total counts and NSB tubes, while 1 ml of 25-OH-D antiserum was added to the tubes labeled standards, controls, and samples. The tubes were mixed gently without foaming and incubated for 90 minutes at 20-25° C. A 500-µl portion of DAG precipitating complex was added to all tubes except the total count tubes. The tubes were mixed and incubated for another 20-25 min at 20-25° C. Additional 500-µl portions of NSB/addition buffer was added to all the tubes except the total count tubes. The tubes were vortexed to mix well and centrifuged for 20 minutes at 20-25° C at 1800 x g, except the total counts tube. A foam rack holder was used to decant the supernate from all the tubes except the total count tubes by inverting the rack into the radioactive waste container. The inverted foam racks were placed onto absorbent paper for 2-3 min; the tubes were blotted gently to ensure that all liquid was removed. The tubes were counted in the gamma counter (Cobra II Auto Gamma Counter 101499, Packard Inc. Norwalk, CT) for 1 minute.

#### 4.3.4 - Bone Strength Test

Mechanical properties of the femur bone were determined by using a three-point bending test with an Instron Universal Testing Instrument (Model 1122, Instron, Canton MA). By applying force to the mid-section of the bone resting on two stationary objects, the force applied was used to determine the strength of the bone (Figure 2).

**Figure 4.1**



The right femur was removed from the rat and cleaned by removing the attached soft tissues and muscles. The bones were then individually sealed in plastic bags and kept at -4° C until ready to use. The bones were thawed at room temperature for the measurements of diameter and length with electronic calipers, and the three-point bending test. The bone thickness was measured with a digital caliper at three different points. Because bone diameter is asymmetrical, an average of these three points was

taken as the diameter of the bone. For bone strength measurement, the bones were rested on two adjustable fulcra, 1.9 cm apart and force was applied to the mid-section of the bone with a plunger moving downward at a rate of 5 mm/min. The maximum shear stress at fracture was used as the indicator of bone strength.

#### **4.4 - Statistical Methods**

Software from SAS (Cary, NC) was used for statistical analysis and a correlation test was performed using the GLM procedure to determine the correlation between calcium in water, calcium in food, vitamin D in water, and vitamin D in diet to other dependent variables. The definition or method of calculation of independent and dependent variables are listed in Table 2. Correlation coefficients for linear regression ( $r^2$ ) were determined after viewing scatter plots using JMP software (SAS, Inc., Cary NC).

Another statistical analysis used in this data analysis was Waller-Duncan K-ratio t Test, a multiple range test that compares Type 1 and Type 2 error rate based on Bayesian principles (Steel and Torrie, 1980). Statistical differences between group means are indicated by absence of a common superscript letter in the data tables.

## **4.5 - Results and Discussion**

**Table 4.2**  
**Term and definition of variables**

| Term used                           | Meaning                                                                                                   |
|-------------------------------------|-----------------------------------------------------------------------------------------------------------|
| <b>Independent variables</b>        |                                                                                                           |
| Calcium intake from diet (mg/wk)    | Diet consumed (g/week) * Calcium concentration in diet (mg/g)                                             |
| Calcium intake from water (mg/wk)   | Weekly water consumption (mL) * calcium concentration in water (mg/mL)                                    |
| Vitamin D intake from diet (µg/wk)  | Weekly food consumed (g) * vitamin D concentration in diet (µg/g)                                         |
| Vitamin D intake from water (µg/wk) | Weekly water consumption (mL) * vitamin D concentration in water (µg/mL)                                  |
| <b>Dependent variables</b>          |                                                                                                           |
| Food intake                         | Average weekly food intake over 5 weeks (g)                                                               |
| Water intake                        | Average weekly water intake over 5 weeks (mL)                                                             |
| Serum Vitamin D                     | Vitamin D concentration detected in serum (ng/mL)                                                         |
| Serum Calcium                       | Calcium concentration in serum (mg/L)                                                                     |
| Protein Serum                       | Protein level or concentration in serum (g/dL)                                                            |
| Bone weight                         | Weight of the dried femur bone (g)                                                                        |
| Bone wall thickness                 | Average measurements of the bone's wall thickness (mm)                                                    |
| Bone length                         | Measurement of the length of the femur bone (mm)                                                          |
| Bone strength                       | The force required to fracture the mid-section of the femur bone at a constant speed (N/mm <sup>2</sup> ) |
| % Calcium in ash                    | Calcium content in bone (mg)/(ash weight (mg)) *100]                                                      |
| Ash weight                          | Weight of the ashed femur bone (g)                                                                        |

### **4.5.1 - Experiment 1, Waller Duncan K-ratio data**

The highest mean Ca intake from diet was from Group D followed by Group J (Table 4.4). The means are groups using the Waller grouping, so Group D rats had the highest concentration of calcium in their diet. Calcium in diet is calculated by multiplying the concentration of calcium in the rat's diet by the amount of food

consumed. Groups M, K, and L had very low food intake. The highest mean Ca intake from water was seen in rat groups I, J, K and, M which did not differ from each other. These groups of rats had the highest concentration of calcium in their water. Groups A, B, C, and Q had no calcium in their water (Table 4.4).

Vitamin D intake from water was approximately ranked according to the vitamin D concentration added to the diet. Groups M and L had lower than expected vitamin D intake because the high  $\text{CaCl}_2$  concentration in their water reduced water volume intake (Table 4.4). Group A had the highest group mean total water intake (Table 4.3). This group had no calcium or vitamin D added to their water. The next highest group rat mean was group B, with no calcium and minimum vitamin D in water, followed by the control group Q with no vitamin D and calcium added to water. There was no significant difference between group B and Q, but there was a significant difference between these two groups and group A. The lower the concentration of calcium in water, the higher the mean water intake.

Group Q had vitamin D formulated into their diet and vitamin D was not added to the water. This group had higher mean serum vitamin D than the other groups, but none of the groups were significantly different, even though the means ranged from 0.307 (Group F) to 6.382 ng/mL (Group Q) (Table 4.5). All of these values were significantly lower than normal serum 25-hydroxy vitamin D concentrations (15-80 ng/mL). Body weight is dependent on adequate water consumption and total food consumed. Group Q had the highest body weight and this could have resulted from the diet formulation as well as the water consumption (Table 4.3). This group mean was significantly different from other groups. Group K, L and M had low water and food intake during the repletion

phase of the study which resulted in weight loss. Due to the weight loss, these rats were sacrificed before the end of the study and final body weights were not compared with the other groups.

Rats with vitamin D deficiency do not necessarily display hypocalcaemia, especially neonatal rats. Lester, et. al (1982) showed that plasma calcium levels did not decrease in spite of low vitamin D concentration in the serum. In our experiment, the group with the lowest serum calcium (group B) was significantly lower than the 7 groups with the highest serum calcium (groups Q, E, J, D, F, I, and C) (Table 4.5). The data suggest that the low serum Ca in group B was due to a drop in the calcium bound to serum protein, rather than a decrease in ionized  $[Ca^{2+}]$ . Serum protein in all groups of rats was only different between the highest group (Group A) and the lowest group (Group B) (Table 4.5).

The rat group that had the highest mean bone weight was group H (Table 4.6). This group had the highest vitamin D concentration and average concentration of calcium added to drinking water. Vitamin D activation increases calcium absorption. Group H was not significantly different from groups Q and E, but was significantly greater than all other groups. None of the other groups differed from each other. Group H did not have a higher serum calcium, serum protein, or serum vitamin D (Table 4.5), or food or water intake than the other groups, so there is no obvious explanation for the higher bone weight. Ash weight was derived from drying and ashing the femur of the rat. The highest mean ash weight was group Q, followed by group H, similar to the bone weight response (Table 4.6).

Group F had the highest mean percentage of calcium present (Table 4.6) in the ash and group H had the lowest mean. The bone wall thickness is asymmetrical and an average of three different measurements is the bone wall thickness. The concentration of vitamin D and calcium does not correlate with these thickness data unlike bone weight data (Table 4.6).

Table 4.3: Food intake, Water intake and Body weight among groups  
Waller Duncan K-ratio t-Test for  
Experiment 1(Normal Calcium, no vitamin D)

| <b>Rat group</b> | <b>Ca in water (g/L)</b> | <b>Vitamin D in Water (µg/L)</b> | <b>Total Food Intake (g)</b> | <b>Total Water Intake (ml)</b> | <b>Final Body weight (g)</b> |
|------------------|--------------------------|----------------------------------|------------------------------|--------------------------------|------------------------------|
| a                | 0                        | 0                                | 375.6 <sup>dc</sup>          | 577.5 <sup>a</sup>             | 386.0 <sup>ed</sup>          |
| b                | 0                        | 7.2                              | 378.7 <sup>bdc</sup>         | 539.3 <sup>ba</sup>            | 409.1 <sup>bcd</sup>         |
| c                | 0                        | 28.8                             | 396.1 <sup>bdac</sup>        | 486.96 <sup>bcd</sup>          | 410.3 <sup>bcd</sup>         |
| d                | 0.72                     | 3.6                              | 438.2 <sup>a</sup>           | 430.70 <sup>ead</sup>          | 400.1 <sup>becc</sup>        |
| e                | 0.72                     | 14.4                             | 414.3 <sup>bac</sup>         | 501.2 <sup>bc</sup>            | 424.1 <sup>ba</sup>          |
| f                | 1.44                     | 0                                | 339.5 <sup>dc</sup>          | 383.4 <sup>ef</sup>            | 403.3 <sup>becc</sup>        |
| g                | 1.44                     | 7.2                              | 354.0 <sup>d</sup>           | 420.3 <sup>ed</sup>            | 415.2 <sup>bc</sup>          |
| h                | 1.44                     | 28.8                             | 391.0 <sup>bdac</sup>        | 432.2 <sup>ead</sup>           | 403.7 <sup>becc</sup>        |
| i                | 2.88                     | 3.6                              | 413.7 <sup>bac</sup>         | 318.5 <sup>gf</sup>            | 387.0 <sup>ecd</sup>         |
| j                | 2.88                     | 14.4                             | 430.8 <sup>ba</sup>          | 298.5 <sup>g</sup>             | 378.0 <sup>e</sup>           |
| k                | 5.76                     | 0                                | 117.9 <sup>e</sup>           | 140.6 <sup>h</sup>             | 0 <sup>f</sup>               |
| l                | 5.76                     | 7.2                              | 102.9 <sup>e</sup>           | 108.3 <sup>h</sup>             | 0 <sup>f</sup>               |
| m                | 5.76                     | 28.8                             | 134.1 <sup>e</sup>           | 137.0 <sup>h</sup>             | 0 <sup>f</sup>               |
| Q                | 0                        | 0                                | 406.6 <sup>bdac</sup>        | 526.9 <sup>ba</sup>            | 445.0 <sup>a</sup>           |

*Group's means of all the factors are listed and significant differences among means (Waller Duncan k-ratio t-Test) are indicated by values not sharing a common superscript letter.*

**Table 4.4: Ca and vitamin D intake among treatment groups**  
**Waller Duncan K-ratio t-Test**  
**Experiment 1(Normal Calcium, no vitamin D)**

| <b>Rat group</b> | <b>Ca intake from Diet (mg/wk)</b> | <b>Ca intake from Water (mg/wk)</b> | <b>Total Ca Intake (mg/wk)</b> | <b>Vitamin D Intake from Diet (µg/wk)</b> | <b>Vit D intake from Water (µg/wk)</b> | <b>Total Vitamin D Intake (µg/wk)</b> |
|------------------|------------------------------------|-------------------------------------|--------------------------------|-------------------------------------------|----------------------------------------|---------------------------------------|
| a                | 1878.2 <sup>dc</sup>               | 0 <sup>d</sup>                      | 1878                           | 0 <sup>b</sup>                            | 0.00 <sup>g</sup>                      | 0.00 <sup>g</sup>                     |
| b                | 1893.3 <sup>bdc</sup>              | 0 <sup>d</sup>                      | 1893                           | 0 <sup>b</sup>                            | 3.88 <sup>ed</sup>                     | 3.88 <sup>ed</sup>                    |
| c                | 1980.4 <sup>bdac</sup>             | 0 <sup>d</sup>                      | 1980                           | 0 <sup>b</sup>                            | 14.02 <sup>a</sup>                     | 14.02 <sup>a</sup>                    |
| d                | 2190.8 <sup>a</sup>                | 310.1 <sup>c</sup>                  | 2501                           | 0 <sup>b</sup>                            | 1.55 <sup>f</sup>                      | 1.55 <sup>f</sup>                     |
| e                | 2071.3 <sup>bac</sup>              | 360.8 <sup>c</sup>                  | 2432                           | 0 <sup>b</sup>                            | 7.22 <sup>c</sup>                      | 7.22 <sup>c</sup>                     |
| f                | 1847.4 <sup>dc</sup>               | 552.0 <sup>b</sup>                  | 2399                           | 0 <sup>b</sup>                            | 0.00 <sup>g</sup>                      | 0.00 <sup>g</sup>                     |
| g                | 1769.8 <sup>d</sup>                | 605.2 <sup>b</sup>                  | 2375                           | 0 <sup>b</sup>                            | 3.03 <sup>d</sup>                      | 3.03 <sup>d</sup>                     |
| h                | 1955.2 <sup>bdac</sup>             | 622.3 <sup>b</sup>                  | 2578                           | 0 <sup>b</sup>                            | 12.45 <sup>b</sup>                     | 12.45 <sup>b</sup>                    |
| i                | 2068.3 <sup>bac</sup>              | 917.4 <sup>a</sup>                  | 2986                           | 0 <sup>b</sup>                            | 1.15 <sup>f</sup>                      | 1.15 <sup>f</sup>                     |
| j                | 2153.9 <sup>ba</sup>               | 859.6 <sup>a</sup>                  | 3014                           | 0 <sup>b</sup>                            | 4.30 <sup>d</sup>                      | 4.30 <sup>d</sup>                     |
| k                | 589.5 <sup>e</sup>                 | 809.9 <sup>a</sup>                  | 1399                           | 0 <sup>b</sup>                            | 0.00 <sup>g</sup>                      | 0.00 <sup>g</sup>                     |
| l                | 514.7 <sup>e</sup>                 | 623.6 <sup>b</sup>                  | 1138                           | 0 <sup>b</sup>                            | 0.78 <sup>gf</sup>                     | 0.78 <sup>gf</sup>                    |
| m                | 670.5 <sup>e</sup>                 | 788.9 <sup>a</sup>                  | 1459                           | 0 <sup>b</sup>                            | 3.95                                   | 3.95 <sup>d</sup>                     |
| Q                | 2033 <sup>bdac</sup>               | 0 <sup>d</sup>                      | 2033                           | 10.16 <sup>a</sup>                        | 0.00                                   | 10.17 <sup>b</sup>                    |

*Group's means of all the factors are listed and significant differences among means (Waller Duncan k-ratio t-Test) are indicated by values not sharing a common superscript letter.*

Table 4.5: Serum vitamin D, Ca and protein values among groups

Waller Duncan K-ratio t-Test

Experiment 1(Normal Calcium, no vitamin D)

| <b>Rat group</b> | <b>Serum Vit. D<br/>(ng/ml)</b> | <b>Serum Calcium<br/>(mg/L)</b> | <b>Serum Protein<br/>(g/100ml)</b> |
|------------------|---------------------------------|---------------------------------|------------------------------------|
| a                | 1.29 <sup>a</sup>               | 83.8 <sup>ba</sup>              | 6.32 <sup>a</sup>                  |
| b                | 0.51 <sup>a</sup>               | 72.9 <sup>b</sup>               | 4.68 <sup>b</sup>                  |
| c                | 1.52 <sup>a</sup>               | 95.6 <sup>a</sup>               | 5.47 <sup>ba</sup>                 |
| d                | 1.54 <sup>a</sup>               | 97.8 <sup>a</sup>               | 5.9 <sup>ba</sup>                  |
| e                | 1.26 <sup>a</sup>               | 98.6 <sup>a</sup>               | 5.82 <sup>ba</sup>                 |
| f                | 0.31 <sup>a</sup>               | 97.1 <sup>a</sup>               | 5.4 <sup>ba</sup>                  |
| g                | 1.58 <sup>a</sup>               | 88.7 <sup>ba</sup>              | 5.66 <sup>ba</sup>                 |
| h                | 1.30 <sup>a</sup>               | 89.0 <sup>ba</sup>              | 5.98 <sup>ba</sup>                 |
| i                | 1.09 <sup>a</sup>               | 98.3 <sup>a</sup>               | 5.84 <sup>ba</sup>                 |
| j                | 0.43 <sup>a</sup>               | 98.3 <sup>a</sup>               | 5.82 <sup>ba</sup>                 |
| k                | 1.39 <sup>a</sup>               | N/A                             | 5.76 <sup>ba</sup>                 |
| l                | 1.19 <sup>a</sup>               | N/A                             | 6.23 <sup>ba</sup>                 |
| m                | 1.28 <sup>a</sup>               | N/A                             | 5.9 <sup>ba</sup>                  |
| q                | 6.38 <sup>a</sup>               | 99.0 <sup>a</sup>               | 6.06 <sup>ba</sup>                 |

*Group's means of all the factors are listed and significant differences among means (Waller Duncan k-ratio t-Test) are indicated by values not sharing a common superscript letter.*

Table 4.6: Measurements on femur bones among rat groups  
Waller Duncan K-ratio t-Test  
Experiment 1(Normal Calcium, no vitamin D)

| <b>Rat group</b> | <b>Bone Weight (mg)</b> | <b>Bone wall thickness (mm)</b> | <b>Bone Length (mm)</b> | <b>Bone Stress (N/mm<sup>2</sup>)</b> | <b>Ash Ca (%)</b> | <b>Ash Weight (mg)</b> |
|------------------|-------------------------|---------------------------------|-------------------------|---------------------------------------|-------------------|------------------------|
| a                | 484.6 <sup>b</sup>      | .687 <sup>bac</sup>             | 32.7 <sup>ed</sup>      | 139.0 <sup>ba</sup>                   | 63.6 <sup>a</sup> | 308.4 <sup>bcd</sup>   |
| b                | 509 <sup>b</sup>        | .781 <sup>a</sup>               | 32.9 <sup>cebd</sup>    | 126.7 <sup>ba</sup>                   | 58.8 <sup>a</sup> | 299.5 <sup>fbecd</sup> |
| c                | 520 <sup>b</sup>        | .747 <sup>ba</sup>              | 32.8 <sup>ced</sup>     | 137.8 <sup>ba</sup>                   | 58.0 <sup>a</sup> | 307.0 <sup>becd</sup>  |
| d                | 520 <sup>b</sup>        | .717 <sup>bac</sup>             | 33.1 <sup>cbd</sup>     | 140.5 <sup>ba</sup>                   | 58.1 <sup>a</sup> | 302.2 <sup>fbecd</sup> |
| e                | 548.2 <sup>ba</sup>     | .639 <sup>bc</sup>              | 34.6 <sup>a</sup>       | 89.6 <sup>c</sup>                     | 57.3 <sup>a</sup> | 314.3 <sup>bc</sup>    |
| f                | 487 <sup>b</sup>        | .590 <sup>c</sup>               | 32.5 <sup>e</sup>       | 113.4 <sup>bc</sup>                   | 64.5 <sup>a</sup> | 292.2 <sup>fed</sup>   |
| g                | 513.6 <sup>b</sup>      | .663 <sup>bac</sup>             | 32.5 <sup>e</sup>       | 142.7 <sup>ba</sup>                   | 57.3 <sup>a</sup> | 294.3 <sup>fecd</sup>  |
| h                | 620.3 <sup>a</sup>      | .706 <sup>bac</sup>             | 33.4 <sup>b</sup>       | 127.7 <sup>ba</sup>                   | 51.0 <sup>a</sup> | 316.5 <sup>ba</sup>    |
| i                | 497.8 <sup>b</sup>      | .747 <sup>ba</sup>              | 32.6 <sup>ed</sup>      | 126.2 <sup>ba</sup>                   | 57.7 <sup>a</sup> | 287.2 <sup>fe</sup>    |
| j                | 503.4 <sup>b</sup>      | .686 <sup>bac</sup>             | 32.8 <sup>cebd</sup>    | 149.6 <sup>a</sup>                    | 56.7 <sup>a</sup> | 285.4 <sup>f</sup>     |
| q                | 549.8 <sup>ba</sup>     | .714 <sup>bac</sup>             | 33.3 <sup>cb</sup>      | 139.1 <sup>ba</sup>                   | 61.1 <sup>a</sup> | 335.7 <sup>a</sup>     |

*Group means of all the factors are listed and significant differences among means (Waller Duncan k-ratio t-Test) are indicated by values not sharing a common superscript letter.*

#### **4.5.2 - Experiment 2, Waller Duncan K-ratio data**

Experiment 2 had been planned as a repetition of Experiment 1 with a lower level of dietary calcium, 2 g Ca/kg diet instead of 5 g Ca/kg of diet. However, given the poor performance of rats given the highest calcium concentration in water (5.76 g/L), Groups K, L and M (Table 1) were not included in Experiment 2. Also, instead of a weekly change of bottled water, fresh water was given to rats every two days to reduce the foul odor detected in water in experiment 1 that could have been caused by bacterial growth. The lower calcium in the diet in Experiment 2 might relate to a common *ad libitum* diet for humans that only includes 40% of the RDA for calcium.

Group Q, the positive control, consumed the most food (Table 4.7). The diet of this group differed from the diet of the other rats in that it had adequate Ca (5 g/kg) and vitamin D. Group D had the highest mean water consumption (Table 4.7). This intake was significantly different from all groups except Q and H. Group J, which had the highest calcium concentration in drinking water consumed significantly less water than groups D, Q, and H (Table 4.7).

Calcium in diet was calculated by multiplying the total food consumed during the study by the concentration of calcium in the food. Group Q had the highest mean calcium intake from diet and was significantly greater than all other groups (Table 4.8). Groups C, G, H, E, F, and J also had significantly higher Ca intake from diet than Group A. Calcium intake from water, calculated by multiplying the total water consumed by the calcium concentration in water, was greatest for Group I, and followed by Group J. These two groups were significantly different from one another, but the calcium concentration in the water was the same. Otherwise, the ranking of calcium intake from

water for the groups was in the same order as the calcium fortification level in the drinking water (Table 4.8).

The only group that had vitamin D in its diet was group Q. As expected, the highest intakes of vitamin D from water were from groups H and group C, which had the highest concentration of vitamin D in their water (Table 4.8).

The mean body weight at the end of the experiment did not significantly differ among groups Q, H, G, E, D, and C (Table 4.7). These groups were significantly heavier than the rats in groups I, B, and A. The data suggest interactions between calcium and vitamin D intake in relation to body weight in this experiment. The ranking of groups for body weight was similar to the ranking for food intake, as expected. Group H has the highest level of calcium and vitamin D, which could result in stronger bones, increasing the rat's body weight. Serum vitamin D for group Q was significantly greater than other groups. The mean of group Q serum vitamin D is approximately 25 times more than other groups, which had serum 25-OH vitamin D concentrations near the lower level of detection (table 4.8). Evidently the vitamin D from water in this study was not available to the rats.

Calcium concentration in serum was higher in Groups I and G than in Groups F and A, suggesting that calcium in water increased the calcium in serum. The serum protein concentration in these groups did not differ; Group B had higher serum protein concentration than Groups J, I, F and A (Table 4.9).

Groups Q, H and I had significantly greater femur weight than did Groups C, A and B. (Table 4.10). Thus the calcium content of the water seems to have increased bone weight, whereas the vitamin D content of the water did not. Lack of bone weight

response to vitamin D would be expected, given that the vitamin D in water did not increase vitamin D content in serum. Ash weight results were very similar to bone weights, as expected. Group Q had the highest mean ash weight (Table 10).

The percent calcium in the ash was as expected. Group B, C, and A had significantly lower ash Ca percentage than did groups H, J, I, D, E, F and G (Table 4.10). Calcium was not formulated into the water of groups B, C and A, unlike the other groups. The femur wall thickness (Table 4.10) and bone length (Table 4.10) were not significantly different among any of the groups. Given the similarity of bone dimensions, the difference in bone mass (Table 4.10) probably reflects true differences in the degree of mineralization.

Bone stress is used to measure the force needed to break a bone at midpoint with a plunger traveling at a constant speed. The force varies with bone strength. Group F had the highest bone strength (significantly different from groups C and A), followed by groups D, Q, G, and J, which had bones significantly stronger than group A. Thus lower calcium intake tended to result in weaker bones than when rats consumed more calcium through fortified drinking water (Table 4.10).

Table 4.7: Food intake, water intake and body weight among groups  
Waller Duncan K-ratio t-Test  
Experiment 2 (Low Calcium, no vitamin D)

| <b>Rat group</b> | <b>Total Food Intake (g)</b> | <b>Total Water Intake (ml)</b> | <b>Ca Conc. in Water (g/L)</b> | <b>Vitamin D Conc. in Water (µg/L)</b> | <b>Final body Weight (g)</b> |
|------------------|------------------------------|--------------------------------|--------------------------------|----------------------------------------|------------------------------|
| a                | 548.4 <sup>d</sup>           | 1299 <sup>bc</sup>             | 0                              | 0                                      | 327.08 <sup>f</sup>          |
| b                | 656.8 <sup>c</sup>           | 1261.9 <sup>bc</sup>           | 0                              | 7.2                                    | 356.82 <sup>fe</sup>         |
| c                | 762.9 <sup>ba</sup>          | 1304.8 <sup>bc</sup>           | 0                              | 28.8                                   | 392.78 <sup>bdac</sup>       |
| d                | 655.0 <sup>c</sup>           | 1798 <sup>a</sup>              | 0.72                           | 3.6                                    | 396.94 <sup>bdac</sup>       |
| e                | 696.5 <sup>bc</sup>          | 1329.7 <sup>bc</sup>           | 0.72                           | 14.4                                   | 399.46 <sup>bdac</sup>       |
| f                | 688.6 <sup>bc</sup>          | 1326.6 <sup>bc</sup>           | 1.44                           | 0                                      | 380.38 <sup>bdec</sup>       |
| g                | 712.2 <sup>bac</sup>         | 1252.4 <sup>bc</sup>           | 1.44                           | 7.2                                    | 410.44 <sup>bac</sup>        |
| h                | 707.4 <sup>bac</sup>         | 1366.2 <sup>ba</sup>           | 1.44                           | 28.8                                   | 413.64 <sup>ba</sup>         |
| i                | 654.2 <sup>c</sup>           | 1180.7 <sup>bc</sup>           | 2.88                           | 3.6                                    | 374.50 <sup>de</sup>         |
| j                | 676.3 <sup>bc</sup>          | 889 <sup>c</sup>               | 2.88                           | 14.4                                   | 379.90 <sup>dec</sup>        |
| q                | 802.7 <sup>a</sup>           | 1447.3 <sup>ba</sup>           | 0                              | 0                                      | 424.90 <sup>a</sup>          |

*Group's means of all the factors are listed and significant differences among means (Waller Duncan k-ratio t-Test) are indicated by values not sharing a common superscript letter.*

**Table 4.8 : Ca and Vitamin D intake among treatment groups**  
**Waller Duncan K-ratio t-Test**  
**Experiment 2 (Low Calcium, no vitamin D)**

| <b>Rat group</b> | <b>Ca intake from Diet (mg/wk)</b> | <b>Ca intake from Water (mg/wk)</b> | <b>Total Ca Intake (mg/wk)</b> | <b>Vitamin D intake from Diet (µg/wk)</b> | <b>Vitamin D Intake from water (µg/wk)</b> | <b>Total Vit. D intake (µg/wk)</b> |
|------------------|------------------------------------|-------------------------------------|--------------------------------|-------------------------------------------|--------------------------------------------|------------------------------------|
| a                | 1097 <sup>c</sup>                  | 0 <sup>e</sup>                      | 1097                           | 0.00 <sup>b</sup>                         | 0.00 <sup>f</sup>                          | 0.00 <sup>f</sup>                  |
| b                | 1314 <sup>cb</sup>                 | 0 <sup>e</sup>                      | 1314                           | 0.00 <sup>b</sup>                         | 9.09 <sup>dc</sup>                         | 9.09 <sup>dc</sup>                 |
| c                | 1526 <sup>b</sup>                  | 0 <sup>e</sup>                      | 1526                           | 0.00 <sup>b</sup>                         | 37.58 <sup>a</sup>                         | 37.58 <sup>a</sup>                 |
| d                | 1310 <sup>cb</sup>                 | 1295 <sup>d</sup>                   | 2605                           | 0.00 <sup>b</sup>                         | 6.47 <sup>de</sup>                         | 6.47 <sup>de</sup>                 |
| e                | 1393 <sup>b</sup>                  | 957 <sup>d</sup>                    | 2350                           | 0.00 <sup>b</sup>                         | 19.15 <sup>b</sup>                         | 19.15 <sup>b</sup>                 |
| f                | 1377 <sup>b</sup>                  | 1910 <sup>c</sup>                   | 3287                           | 0.00 <sup>b</sup>                         | 0.00 <sup>f</sup>                          | 0.00 <sup>f</sup>                  |
| g                | 1424 <sup>b</sup>                  | 1803 <sup>c</sup>                   | 3228                           | 0.00 <sup>b</sup>                         | 9.02 <sup>dc</sup>                         | 9.02 <sup>dc</sup>                 |
| h                | 1415 <sup>b</sup>                  | 1967 <sup>c</sup>                   | 3382                           | 0.00 <sup>b</sup>                         | 39.35 <sup>a</sup>                         | 39.35 <sup>a</sup>                 |
| i                | 1308 <sup>cb</sup>                 | 3400 <sup>a</sup>                   | 4709                           | 0.00 <sup>b</sup>                         | 4.25 <sup>fe</sup>                         | 4.25 <sup>fe</sup>                 |
| j                | 1353 <sup>b</sup>                  | 2560 <sup>b</sup>                   | 3913                           | 0.00 <sup>b</sup>                         | 12.80 <sup>c</sup>                         | 12.80 <sup>c</sup>                 |
| q                | 4013 <sup>a</sup>                  | 0 <sup>e</sup>                      | 4013                           | 20.07 <sup>a</sup>                        | 0.00 <sup>f</sup>                          | 20.07 <sup>b</sup>                 |

*Group's means of all the factors are listed and significant differences among means (Waller Duncan k-ratio t-Test) are indicated by values not sharing a common superscript letter*

Table 4.9 : Serum Vitamin D, Calcium and Protein values among groups

Waller Duncan K-ratio t-Test

Experiment 2 (Low Calcium, no vitamin D)

| <b>Rat group</b> | <b>Serum Calcium (mg/L)</b> | <b>Serum Protein (g/100ml)</b> | <b>Vit D Serum (ng/ml)</b> |
|------------------|-----------------------------|--------------------------------|----------------------------|
| a                | 71.15 <sup>c</sup>          | 5.62 <sup>b</sup>              | 1.26 <sup>b</sup>          |
| b                | 93.15 <sup>bac</sup>        | 6.98 <sup>a</sup>              | 1.67 <sup>b</sup>          |
| c                | 98.55 <sup>ba</sup>         | 6.52 <sup>ba</sup>             | 2.47 <sup>b</sup>          |
| d                | 104.2 <sup>ba</sup>         | 6.56 <sup>ba</sup>             | 1.45 <sup>b</sup>          |
| e                | 99.5 <sup>ba</sup>          | 6.42 <sup>ba</sup>             | 1.72 <sup>b</sup>          |
| f                | 78.35 <sup>bc</sup>         | 5.92 <sup>b</sup>              | 1.76 <sup>b</sup>          |
| g                | 106.25 <sup>a</sup>         | 6.44 <sup>ba</sup>             | 2.03 <sup>b</sup>          |
| h                | 98.40 <sup>ba</sup>         | 6.22 <sup>ba</sup>             | 2.39 <sup>b</sup>          |
| i                | 114.75 <sup>a</sup>         | 5.94 <sup>b</sup>              | 1.95 <sup>b</sup>          |
| j                | 104.8 <sup>ba</sup>         | 5.98 <sup>b</sup>              | 2.16 <sup>b</sup>          |
| q                | 101.40 <sup>ba</sup>        | 6.18 <sup>ba</sup>             | 52.93 <sup>a</sup>         |

*Group's means of all the factors are listed and significant differences among means (Waller Duncan k-ratio t-Test) are indicated by values not sharing a common superscript letter*

Table 4.10 : Measurements on femur bones among rat groups

Waller Duncan K-ratio t-Test

Experiment 2 (Low Calcium, no vitamin D)

| <b>Rat group</b> | <b>Bone Length (mm)</b> | <b>Bone Wall Thickness (mm)</b> | <b>Bone Stress</b>    | <b>Bone Weight (mg)</b> | <b>Ash Ca (%)</b>   | <b>Ash Weight (mg)</b> |
|------------------|-------------------------|---------------------------------|-----------------------|-------------------------|---------------------|------------------------|
| a                | 32.02 <sup>a</sup>      | .619 <sup>a</sup>               | 91.05 <sup>c</sup>    | 457.66 <sup>e</sup>     | 55.74 <sup>a</sup>  | 255.1 <sup>d</sup>     |
| b                | 32.77 <sup>a</sup>      | .689 <sup>a</sup>               | 118.51 <sup>bac</sup> | 449.60 <sup>e</sup>     | 56.98 <sup>a</sup>  | 256.18 <sup>d</sup>    |
| c                | 31.55 <sup>a</sup>      | .681 <sup>a</sup>               | 118.01 <sup>bac</sup> | 468.52 <sup>ed</sup>    | 56.56 <sup>a</sup>  | 265.02 <sup>d</sup>    |
| d                | 33.05 <sup>a</sup>      | .755 <sup>a</sup>               | 135.43 <sup>ba</sup>  | 513.48 <sup>bc</sup>    | 59.03 <sup>a</sup>  | 303.10 <sup>cb</sup>   |
| e                | 32.41 <sup>a</sup>      | .682 <sup>a</sup>               | 107.90 <sup>bc</sup>  | 522.44 <sup>bc</sup>    | 58.00 <sup>a</sup>  | 303.00 <sup>cb</sup>   |
| f                | 33.14 <sup>a</sup>      | .685 <sup>a</sup>               | 146.71 <sup>a</sup>   | 503.74 <sup>c</sup>     | 57.99 <sup>a</sup>  | 292.14 <sup>c</sup>    |
| g                | 33.45 <sup>a</sup>      | .703 <sup>a</sup>               | 131.23 <sup>ba</sup>  | 519.86 <sup>bc</sup>    | 58.03 <sup>aa</sup> | 301.70 <sup>cb</sup>   |
| h                | 32.88 <sup>a</sup>      | .742 <sup>a</sup>               | 118.60 <sup>bac</sup> | 538.74 <sup>ba</sup>    | 58.34 <sup>a</sup>  | 314.28 <sup>b</sup>    |
| i                | 32.81 <sup>a</sup>      | .732 <sup>a</sup>               | 119.72 <sup>bac</sup> | 529.26 <sup>bac</sup>   | 57.71 <sup>a</sup>  | 305.44 <sup>cb</sup>   |
| j                | 32.68 <sup>a</sup>      | .742 <sup>a</sup>               | 126.74 <sup>ba</sup>  | 498.48 <sup>dc</sup>    | 58.61               | 292.14 <sup>c</sup>    |
| q                | 32.50 <sup>a</sup>      | .719 <sup>a</sup>               | 135.15 <sup>ba</sup>  | 563.78 <sup>a</sup>     | 60.06 <sup>a</sup>  | 338.60 <sup>a</sup>    |

*Group means of all the factors are listed and significant differences among means (Waller Duncan k-ratio t-Test) are indicated by values not sharing a common superscript letter*

#### **4.5.3 - Experiment 1 correlation data (Normal calcium and no vitamin D study)**

As shown in Table 4.11, there was a significant correlation ( $P = 0.015$ ) between total calcium intake (Ca intake from diet plus Ca intake from water) and serum calcium concentration. None of the other parameters of bone or Ca metabolism shown in Table 4.11 were significantly correlated with either total Ca intake or Ca intake for water alone. Data from Groups K, L, M, and Q were excluded from these analyses. In experiment 1, Groups K, L, and M had the highest amount of calcium in their water, the lowest body weight, and the lowest total water consumption. The rats in these groups drank less water, which resulted low food intake and weight loss, probably due to aversion to the taste of the high calcium chloride concentration in water, and resulting dehydration. Because they were not continued to the end of the study, data comparable to other groups was not available. Group Q (positive control diet) was excluded for the regression analyses to be consistent with Experiment 2, which had a lower dietary Ca. Serum vitamin D was higher in group Q, which had vitamin D in the diet, than in the groups that received vitamin D in water. Preliminary analyses including group Q in the dataset only showed significant positive correlations for Ca intake from water with ash calcium percentage ( $P = 0.0258$ ) and total vitamin D intake with bone length ( $P = 0.0412$ ). Bone length was also significantly correlated ( $P = 0.0411$ ) with total vitamin D intake when Group Q was excluded from the analyses (Table 4.11).

#### **4.5.4 - Experiment 2 correlation data (Low calcium and no vitamin D study)**

In Experiment 2 rats were fed a diet containing only 40% of recommended dietary Ca (2 g/kg rather than 5 g/kg). Both the total calcium intake (food and water) and the

calcium intake from drinking water had significant positive linear correlation with body weight, serum calcium concentration, bone mass, bone ash weight, bone diameter, bone length, and bone breaking strength (Table 4.12). The percentage of Ca in bone ash was negatively correlated with both the total calcium intake (food and water) and the calcium intake from drinking water ( $P = 0.0041$  and  $P = 0.0031$ , respectively). The total vitamin D intake (all from water) was significantly correlated with body weight and serum vitamin d concentration (Table 4.12). Because the serum vitamin D concentration was so low in these rats, it is not surprising that the variation in intake did not significantly affect bone parameters. However, the correlation between vitamin D intake and serum vitamin D suggests that the radioimmunoassay for serum vitamin D was accurate. In previous unpublished studies, acute intragastric infusion of BLG-vitamin complex in water had high bioavailability. In this study, breakdown of the complex and loss of the vitamin D in the rats' water bottles seems a likely explanation for the low vitamin D levels in the rats' sera.

Body weight gained during the study correlated with calcium intake from the diet, calcium intake from water and vitamin D intake (Table 4.12). Low calcium availability apparently impairs growth. Correcting the deficiency either through calcium supplementation or vitamin D supplementation in drinking water can improve the growth response. In contrast, when dietary calcium intake was adequate (Experiment 1), fortification of drinking water with calcium or vitamin D had little effect of rat growth or any parameters of bone metabolism (Table 4.12).

**Table 4.11** Correlation Coefficients ( $r^2$ ) for Experiment 1 (Normal Calcium, no vitamin D in diet). Animals fed the positive control diet were excluded from the analysis.

| 1 <sup>st</sup> Experiment (N) | Total Ca Intake | Ca Intake from Water | Total Vitamin D Intake |
|--------------------------------|-----------------|----------------------|------------------------|
| Body Weight                    | 0.0015          | 0.0259               | 0.0153                 |
| Serum Vitamin D                | 0.0005          | 0.0043               | 0.0093                 |
| Serum Calcium                  | 0.1194*         | 0.0415               | 0.0002                 |
| Serum Protein                  | 0.0318          | 0.0187               | 0.0018                 |
| Bone Weight                    | 0.0267          | 0.0000               | 0.0608                 |
| Ash Weight                     | 0.0233          | 0.0700               | 0.0744                 |
| % Ca in Ash                    | 0.0015          | 0.0081               | 0.0025                 |
| Bone Diameter                  | 0.0112          | 0.0065               | 0.0198                 |
| Bone Length                    | 0.0080          | 0.0033               | 0.0840*                |
| Bone Strength                  | 0.0065          | 0.0002               | 0.0022                 |

- \* =  $P < .05$
- \*\* =  $P < .01$
- \*\*\* =  $P < .001$
- \* or \*\* indicates significant correlation; with no asterisk, the linear model is not significantly better than the mean value

**Table 4.12** Correlation Coefficients ( $r^2$ ) for Experiment 2 (Low Calcium, no vitamin D in diet). Animals fed the positive control diet were excluded from the analysis.

| 2 <sup>nd</sup> Experiment (L) | Total Ca Intake       | Ca Intake from Water  | Total Vitamin D Intake |
|--------------------------------|-----------------------|-----------------------|------------------------|
| Body Weight                    | 0.1181*               | 0.0856*               | 0.1173*                |
| Serum Vitamin D                | 0.0219                | 0.0117                | 0.1930**               |
| Serum Calcium                  | 0.2593***             | 0.2001**              | 0.0106                 |
| Serum Protein                  | 0.0295                | 0.0544                | 0.0521                 |
| Bone Weight                    | 0.3147***             | 0.3128***             | 0.0061                 |
| Ash Weight                     | 0.3971***             | 0.3874***             | 0.0058                 |
| % Ca in Ash                    | 0.1654** <sup>1</sup> | 0.1745** <sup>1</sup> | 0.0254                 |
| Bone Diameter                  | 0.1101*               | 0.0924*               | 0.0201                 |
| Bone Length                    | 0.1418**              | 0.1347*               | 0.0409                 |
| Bone Strength                  | 0.1103*               | 0.0919*               | 0.0037                 |

<sup>1</sup>Significant negative correlation. %Ca in Ash =  $14.59 - 0.00064 * \text{Ca intake}$ ;  
 %Ca in Ash =  $13.76 - 0.0006 * \text{Ca intake from water}$ .

#### **4.5.5 - Experiment 1 general discussion**

During experiment 1, several errors were made that might have affected the results. Drinking water solutions were prepared in 2-L brown plastic bottles and kept at 2-4° C. The water was then transferred to clear glass bottles filled to the top and given to the rats *ad libitum*. The volume of water added was recorded. After 2-3 days water consumption, more water was added to refill the clear bottles. At the end of the week, all drinking solutions were discarded, including water in the clear bottles and brown bottles.

A foul smell was detected in the brown bottles and clear bottles after 3 days and especially with the bottles of water that had high vitamin D. Furthermore, water fed to rats in groups K, L and M (see table 4.1) had the highest level of calcium. As explained above, the rats in groups K,L, and M drank less water and ate less, resulting in a dramatic weight loss. Due to the depressed state of the rats, these groups of rats were sacrificed early.

#### **4.5.5a - Microbiological Test**

The foul smell and the early sacrifice of the rats compromised our results; a microbiological test was performed on some samples of foul smelling water. Toward the end of the study, we hypothesized that bacterial breakdown of BLG used to bind vitamin D might be resulting in the foul odor. An aerobic plate count was performed on drinking water solution from three rat groups stored in the brown bottle. One ml of solution was plated on Petrifilm aerobic count plates (3M Microbiology, St. Paul, MN) and incubated at 25° C. After day 1, some colonies were formed on the plates and after day 6, most of the Petrifilms were filled with colonies. This result (Table 4.13) shows increasing

bacterial growth in the water from day 1 to day 6. The growth of bacteria in the rat water could be breaking down the BLG-vitamin D complex, therefore reducing the vitamin D availability to the rats.

**Table 4.13 Bacterial counts associated with BLG-vitamin D-fortified water fed to selected groups of rats,**

| DAYS #      | GROUP H           | GROUP E           | GROUP C            | DI-WATER           |
|-------------|-------------------|-------------------|--------------------|--------------------|
| D1 (cfu/ml) | $4.5 \times 10^1$ | $1.2 \times 10^3$ | $2.13 \times 10^2$ | $<1.0 \times 10^0$ |
| D6 (cfu/ml) | *TNTC             | *TNTC             | *TNTC              | $1.1 \times 10^1$  |

*\*TNTC = Too numerous to count*

#### **4.5.6 - Experiment 2 general discussion**

In experiment 2, rats were fed a low calcium, vitamin D-deficient diet, and drinking water supplemented with varying concentrations of  $\text{CaCl}_2$  to increase calcium intake. Unlike experiment 1, bacterial contamination of drinking water was not evident from odor in experiment 2. Changing the rat's water every two days helped reduce any bacterial contamination. Also, Groups K, L and J were not included in this study due to the poor performance and body condition of rats in these groups seen in experiment 1.

Supplementation of calcium is recommended when calcium consumption is low. In this study, groups A and B and sometimes group C, tended to have lower means in most of the indicators of calcium utilization that were measured. Group A, which had no supplemental calcium in water, had the lowest mean in all variables except water consumption. Not supplementing these rats' diet or water resulted in the lowest mean

bone strength. Bone stress is the amount of force required to fracture the mid-section of the femur bone. The rats in other groups that were supplemented with  $\text{CaCl}_2$  in water had stronger bones and greater final body weight.

#### **4.6 - Conclusion**

In experiment 1, due to the unforeseen error, three groups of rats had to be sacrificed early and this affected the experiment's result. But these treatment groups were not included in Experiment 2. The taste of the high calcium water may have been unpleasant to the rats, which led to low water consumption and low food intake. However, the study shows that the group of rats with no water supplementation of calcium and vitamin D (Group A), had the weakest bones and lowest bone ash weight. With the low calcium diet in Experiment 2, fortification of water with calcium chloride significantly increased bone strength and mineralization. Fortifying drinking water with calcium chloride significantly reduced the effect of low dietary Ca intake, but had little effect when dietary calcium was adequate.

#### **4.7 - References**

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## Appendices

Name \_\_\_\_\_

Date \_\_\_\_\_

### THRESHOLD TEST BALLOT

You will receive 5 sets of samples, one at a time. Three samples are in each of the five rows and each row is an individual triangle test. Beginning with the row closest to the taped end of the tray, taste the samples in each row from left to right. Swirl each sample in the mouth. Use the provided rinse water to refresh your palate between samples. Once you have completed one row of samples, determine which sample is different (odd) from the other two and write that sample code in the appropriate blank. Proceed to the remaining rows. Even if you cannot identify the different sample, you **MUST GUESS**.

|       | <u>Different Sample</u> | <u>Are you sure?</u><br><u>(Yes/No)</u> |
|-------|-------------------------|-----------------------------------------|
| Set 1 | _____                   | _____                                   |
| Set 2 | _____                   | _____                                   |
| Set 3 | _____                   | _____                                   |
| Set 4 | _____                   | _____                                   |
| Set 5 | _____                   | _____                                   |

What does the sample taste like? \_\_\_\_\_

**THANK YOU!**

**Appendix 1. Threshold test ballot**

## WATER QUESTIONNAIRE

**Please check the appropriate answer for the following demographic information**

1. Sex ☐ male ☐ female

2. Age group

- ☐ 18 years or younger
- ☐ 19 – 25 years
- ☐ 26 - 35 years
- ☐ 36-45 years
- ☐ 46-65 years
- ☐ 65 years or older

3. I am a US citizen ☐ yes ☐ no

Please answer the following questions. There is no right or wrong answers.

4. Do you shop for your household, even if it is you alone? ☐ yes ☐ no

5. Do you purchase fortified products? ☐ yes ☐ no

6. What kind of calcium fortified beverages do you consume? Circle all that apply.

|                      |                 |                    |
|----------------------|-----------------|--------------------|
| a. Milk              | c) Water        | e) other (specify) |
| b. Orange Juice (OJ) | d. Juice drinks |                    |

7. What other kinds of calcium fortified food (not just calcium containing) do you consume?

|                     |                          |                      |
|---------------------|--------------------------|----------------------|
| a. Yogurt           | f. Soymilk/Soy beverages | k. Cheese            |
| b. Waffles/Pancakes | g. Candy                 | l. Frozen Yogurts    |
| c. Cereals          | h. Pasta                 | m. Spread/ Margarine |
| d. Snack Foods      | i. Bread                 |                      |
| e. Tofu             | j. Energy Bars           |                      |

8. How often do you consume any type of calcium fortified products (Circle only one answer)

- a. I do not consume calcium fortified products
- b. Less than once per month
- c. 2 to 4 times per month
- d. More than once a week
- e. Twice a week
- f. Three or more times a week

9. What kind of beverage do you consume? Circle all that apply:

- a. Water (Bottled)
- b. Soda (Pop)
- c. Flavored Water (EX Fruit H2O)
- d. Sport Drinks (Ex. Gatorade, Powerade)
- e. Energy Drinks (Ex. Red Bull)
- f. Tea/coffee
- g. Fruit Juices

10. What is your level of interest in calcium fortified products? Please circle one answer:

- a. Very interested
- b. Somehow interested
- c. Interested
- d. Somehow not interested
- e. Not interested

11. Please indicate how you feel about the following statement”

**“Recommended calcium intake can help prevent Osteoporosis and helps build stronger bones”**

\_\_\_\_\_Agree strongly

\_\_\_\_\_Agree

\_\_\_\_\_Neither Agree or Disagree

\_\_\_\_\_Disagree

\_\_\_\_\_Disagree strongly

11. What factors influence your choice of calcium fortified products? Check all that apply:

\_\_\_\_\_Price

\_\_\_\_\_Availability

\_\_\_\_\_Taste

\_\_\_\_\_Vegan/Lactose Intolerant (need calcium in diet)

\_\_\_\_\_Brand identity

\_\_\_\_\_Don't like milk or dairy products

## **Appendix 2. Water consumer questionnaire**

Samples \_\_\_\_\_

Thirst Quenching Potential

|                      |   |   |   |   |                       |   |   |   |
|----------------------|---|---|---|---|-----------------------|---|---|---|
| 1                    | 2 | 3 | 4 | 5 | 6                     | 7 | 8 | 9 |
| Not Thirst Quenching |   |   |   |   | Very Thirst Quenching |   |   |   |

Overall Flavor Liking

|           |   |                          |   |   |   |   |                |   |
|-----------|---|--------------------------|---|---|---|---|----------------|---|
| 1         | 2 | 3                        | 4 | 5 | 6 | 7 | 8              | 9 |
| Dislike   |   | Neither like nor dislike |   |   |   |   | Like Extremely |   |
| Extremely |   |                          |   |   |   |   |                |   |

Overall Flavor Liking

|           |   |                          |   |   |   |   |                |   |
|-----------|---|--------------------------|---|---|---|---|----------------|---|
| 1         | 2 | 3                        | 4 | 5 | 6 | 7 | 8              | 9 |
| Dislike   |   | Neither like nor dislike |   |   |   |   | Like Extremely |   |
| Extremely |   |                          |   |   |   |   |                |   |

Likes

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Dislikes

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**Appendix 3. Acceptance test ballot**