ABSTRACT

LOVETT, MALLORYE DELORIS. Beta-lactoglobulin Complexed Vitamin D in an Aqueous Solution: Formulation and Bioavailability. (Under the direction of: Jonathan C. Allen).

Growing awareness of vitamin D status in the United States has resurfaced due to increasing reports of insufficiency and deficiency. Epidemiological research indicates that average intake of this nutrient is well below the RDA, and greater intake has been linked to optimal bone health, prevention of osteoporosis, osteomalacia, rickets, and other chronic diseases.

The first objective of this research was to test a water-soluble form of vitamin D as a fortifying ingredient in a flavored aqueous solution. Vitamin D was extracted by organic solvents and assayed by High Performance Liquid Chromatography (HPLC). The second objective was to test the ability of different vitamer sources (D$_2$ versus D$_3$) and quantitative amounts to increase serum vitamin D levels in a human intervention study. Vitamin D content of the complex was assessed by HPLC and VitaKit D™. Flavored beverages were formulated with 400 IU, 600 IU, and 1000 IU of vitamin D. Human subjects recruited from North Carolina State University (Raleigh, NC) participated in a blind randomized clinical trial designed to determine the bioavailability of vitamin D$_2$ versus D$_3$ at various levels. Subjects consumed 8 fl. oz of a vitamin D beta-lactoglobulin complex-fortified beverage at each trial (total 5 trials) and blood was drawn at baseline, 4, 8, 24 hours post-consumption. Serum 25-hydroxy vitamin D was measured by ELISA. Results show that aqueous sports drink solutions could be accurately formulated to contain vitamin D (400 IU, 600 IU, and 1000 IU) with palatability to humans. Serum vitamin D analysis demonstrated a significant difference ($p<0.0001$) in serum vitamin D levels by race. Moreover, there was an increase in blood serum levels for all participants from
initial treatment to final treatment. Subjects did not respond differently to drinks containing vitamin D$_2$ or D$_3$.

In conclusion, water-soluble vitamin-D can be used to fortify aqueous products and consumption can help facilitate the uptake of vitamin into the blood. Regular consumption of flavored drinks fortified with vitamin D can significantly increase dietary vitamin D intake.
Beta-lactoglobulin Complexed Vitamin D in an Aqueous Solution: Formulation and Bioavailability

by
Mallorye Deloris Lovett

A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Food Science

Raleigh, North Carolina
2010

APPROVED BY:

Jonathan C. Allen, PhD
Committee Chair

Brenda P. Alston-Mills, PhD

Leon C. Boyd, PhD

Gabriel Keith Harris, PhD
DEDICATION

This dissertation is dedicated to my parents, sister, grandmother, and in loving memory of my grandparents for their love, sacrifices, life lessons, encouragement, support and prayers, during my academic journey.
BIOGRAPHY

Mallorye D. Lovett was born in Raleigh, North Carolina. She is the youngest daughter of Gus and Gloria Lovett. Mallorye was educated in Wake County Public Schools (Raleigh, NC). She obtained her Bachelor's degree from North Carolina Central University in Durham, NC; she graduated with a Human Sciences degree with a concentration in nutrition/dietetics. During her undergraduate program, Mallorye participated in undergraduate research and attended the AGEP (Alliances in Graduate Education and the Professoriate) Summer Pre-Graduate Research Experience at the University of North Carolina at Chapel Hill. She went on to receive a Master of Science degree in Nutrition from the University of North Carolina at Greensboro. While at UNC-G, she also completed her dietetic internship. After completion of her dietetic internship rotations and graduation, Mallorye was accepted into the graduate program in the Department of Food Science at North Carolina State University (Raleigh, NC) under the advisory of Dr. Jonathan C. Allen in January 2005. While attending North Carolina State University, Mallorye received a Master of Science degree in Food Science and was inducted into the Phi Tau Sigma (Honor Society for Food Scientists). During her graduate studies, Mallorye worked as a summer intern at Kraft Foods, Inc. in Tarrytown, NY and completed requirements to become a Registered Dietitian. After graduating in 2007, she continued her studies at NC State University, to pursue her PhD in Food Science with a minor in Food Safety. Mallorye’s research has focused on protein-vitamin D fortification in aqueous drinks.
ACKNOWLEDGMENTS

The journey from conceptualization to ultimate completion of this degree and research study has been a true odyssey of discovery. An odyssey of this nature and complexity could not have been accomplished without the help, support, and cooperation of many individuals.

Acknowledgements to individuals will be given in no particular order.

• This journey could have not been completed without the love and faithfulness of God.
  “Great is thy faithfulness, Lord unto me” ~ Thomas O. Chisholm; these words have uplifted me through the trails, tribulations and joys of this journey. I am glad to behold His love, grace and faithfulness.

• Dr. Allen thank you for the opportunity to become a member of your lab group.
  Thank you for your continued encouragement and guidance. This opportunity has allowed me to grow in research and teaching.

• My committee members, thank you for your support and advice I am truly grateful.

• The Allen lab group, thank you for encouragement and research advice.

• Paige Luck, Jessica Childs, SAS consulting services personnel, Judy Cooper, Shirley Lyles, and Sabrina Whitley-Ferrell thank you for assistance in equipment utilization, development of computerized questionnaire, analysis of data and overall assistance.
  Each and everyone has been a tremendous support.

• To the participants who were involved in the research study, thank you. The completion of the human study would have not been possible without you.
• To the Southeast Dairy Foods Research Center (SDFRC) and Dairy Management, Inc., for their financial support.

• To the members of the Department of Food, Bioprocessing and Nutrition Sciences, the Food Science Club, and the NCSU Chapter of MANRRS (Minorities in Agriculture, Natural Resources, and Related Science) thank you.

• Montreka, JeVelle, Tristan, Paula, Jeffrey, Jada, Mallory, Holly, Megan, Miniayah, Carmel, Felita, Nhora, Lenese, YieHui, Lei, Lindsey, Wanida and Chellani thank you for all the good times. I will always remember the laughter and great conversations.

• My parents, words cannot express how thankful I am to have outstanding parents like you. You have instilled in me the importance of education and achieving your heart’s desires. Mom thank you for your unremitting encouragement and assistance. Daddy, thank you for continuous mantra of hope and reminding me that I am always loved. Thank you both for your constant love and support.

• My sister, whether near (USA) or far (another continent) you have always been my number one cheerleader. You have encouraged me beyond measure to succeed and never be afraid to go after your dreams. Thanks for your calming disposition and love.

“No man is an island, entire of itself; every man is a piece of the continent.” – John Donne

To my continent of supporters, I say thank you for accompanying me on this journey. To each and every member of the following families and organizations you have contributed to my successes. In no particular order:
• Lovett, Durham, Nixon, Perry, Stiff, Young, Spinks, Crisp, Scott, Clegg, Johnson, 
  Bynum, Brown, Gause, Roberts, Cheatham, Myers, Sanders, Vanderkamp, Sult, 
  Woodard, Hart, Wright, Kearney, Taylor, Flood, Little, friends and family at NCCU, 
  friends of UNC-G, First Baptist Church family, Alpha Lambda Fall 99-94 Degrees 
  and Rising, Delta Sigma Theta Sorority, Inc., P&G RTCl (Research in Technical 
  Careers and Industry) conference cohorts, NC Health Careers – Wake AHEC, Delta 
  Carousel, Top Teens of America, NC-MSEN Pre-College Program, ACTS, and 
  Bennett College Intensive Summer Science Program (ISSP) . If any family or 
  organization was omitted by oversight, it is not meant to indicate that your role was 
  not significant please accept my heartfelt apology for the omission.
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CHAPTER 1

Introduction

Mallorye D. Lovett

Department of Food, Bioprocessing and Nutrition Sciences

North Carolina State University
1.1 Preface

Vitamin D is an essential micronutrient that regulates calcium and phosphorus metabolism to achieve homeostasis and is required for growth, development, and structural integrity of the skeleton. Moreover, vitamin D has non-calcitropic autocrine and paracrine functions that have been shown to play a possible role in cancer and other chronic disease such as diabetes and cardiovascular disease (Holick, 2003). Vitamin D is referred to as the “sunshine vitamin” due to the production vitamin D from cholesterol via photosynthesis when skin is exposed to ultraviolet B light (Holick, 2003, Stechschulte et al., 2009). Vitamin D can also be obtained from various dietary sources including fish liver oils, the flesh of fatty fish, and eggs from hens fed vitamin D enriched diet (IOM, 1997). In the United States the major sources of vitamin D are fortified foods including milk, margarine, breads and fortified breakfast cereals. Milk is the major source of vitamin D from food. Fortified foods account for the majority of vitamin D in the American diet (ODS, 2008). Even though vitamin D can be obtained from dietary sources and photosynthesis via the skin, vitamin D deficiency is now recognized as a pandemic, making it an historic global health event (Stechschulte et al., 2009). This global pandemic notation may be due to the decreased sun exposure in current human lifestyles and a decline in consumption of foods containing vitamin D. Prior to the industrialization period vitamin D status among individuals exposed to sunlight was adequate. Moreover, archeological records have illustrated that humans exposed to adequate sunlight were able to obtain the daily vitamin D requirements for bone development (Holick, 1992, Holick, 1995, Holick, 1999).
Beta-lactoglobulin (β-LG) has many functional properties including the capacity to bind fat-soluble compounds including vitamins A and D. Lipophilic vitamins bind to the hydrophobic core of the β-LG molecule. Therefore using β-LG as a carrier for fortification of low or non-fat solutions could structurally protect vitamins A and D and improved their solubility and stability. Wang et al. (1997) illustrated via fluorescence spectroscopy that β-LG is capable of binding at approximately two moles of retinyl palmitate and one mole of vitamin D per mole of protein. Furthermore, previous studies have suggested that β-LG has a higher affinity for vitamin D than for other lipophilic vitamins.

A possible use for the complex formed between β-LG and vitamin D is fortification of bottled water and similar rehydration products. The bottled water market continues to grow at a faster rate than other beverage sectors. Consumers are aware of the health benefits associated with daily consumption of fluid water. Fortification of bottled water with nutrients (β-LG and vitamin D), non-caloric sweetener, and flavor may increase the bottled water market.

In this study, the bioavailability of β-LG-complexed vitamin D in an aqueous solution was investigated to justify the potential for using β-LG as a stable and protective carrier for vitamin D. The purpose of this study was to produce a beverage fortified with vitamin D in a β-LG complex, demonstrate that the β-LG and vitamin D remain in association with each other in the aqueous environment, and to assess the capacity the protein-based aqueous solution to elevate serum vitamin D status in a human feeding trial.

This dissertation reviews the metabolism and biological functions of vitamin D. Moreover, this dissertation demonstrates the binding ability of β-lactoglobulin with vitamin D. Vitamin D binding to β-lactoglobulin will be assessed by analytical fluorescence spectroscopy testing. The
main body of this document will describe preparation of protein-vitamin complexes, testing for milk fortification compliance, and the bioavailability study. Included throughout the document are the methodologies, results, discussions and conclusion of each experiment.
REFERENCES


CHAPTER 2

Literature Review

Mallorye D. Lovett

Department of Food, Bioprocessing and Nutrition Sciences
North Carolina State University
2.1 Introduction

2.1.1 Water

The most abundant constituent in the human body is water. The body is comprised of an estimated 60 to 75 percent water (ranging from about 50% in obese individuals and 70% in lean individuals). The human brain (95%), lungs (90%), and blood (82%) are composed mainly of water. Water is the cornerstone in the maintenance of good health. Water provides fundamental nutrients necessary to sustain cellular homeostasis and life. Homeostasis in all of the major organ systems in the human body depend on water for the following functions:

- Moistens tissues such as those in the mouth, eyes, and nose
- Protects the body’s organs and tissues
- Aids in digestion
- Helps prevent constipation
- Helps dissolve minerals and other nutrients to increase bioavailability to the body
- Regulates body temperature
- Lubricates joints
- Lessens the burden on the kidneys and liver by flushing out waste products
- Carries nutrients and oxygen to cell

In addition, water is the medium and sometimes a substrate for various enzymatic and chemical reactions that occur in the body. Water has the ability to perform these functions because water crosses cell membranes freely due to osmosis, the flow of water from areas of low solute concentration to areas of high solute concentration (Ritchie, 2007). Thus, water...
facilitates the movement of nutrients, hormones, antibodies, and oxygen throughout the bloodstream and lymphatic system (WHO, 2006). Water utilized by the body is obtained from various sources including drinking water, water in beverages, water obtained from food, and water produced by metabolic reactions. Previous research studies have found an association between low intake of total water and some chronic diseases including breast cancer, prostate cancer, and cardiovascular disease (IOM, 2004). The Institute of Medicine advised that men consume approximately 3 liters (about 13 cups) and women consume 2.2 liters (about 9 cups) of total beverages daily to obtain the beneficial effects of water. For example, the DRI (Dietary Reference Intake) has an AI (Adequate Intake) for total water intake for young men and women (ages 19 to 30 years) of 3.7 L and 2.7 L per day, respectively. These amounts represent approximately 81% of total water intake in the US. Even though the essential benefits of water have been clearly defined, the current United States Department of Agriculture (USDA) MyPyramid does not include water as a component of many foods in their database (WHO, 2005). Although it has been clearly defined that water is the most abundant component in the human body, and despite knowledge of health hazards associated with lack of water, the scientific community is unable to clearly define the definition of optimal water requirement and establish intake recommendations (Ritz, 2005). However, the body of research has established that water is essential to sustain life (IOM, 2004).

2.1.2 Hydration Status

Water homeostasis is maintained within narrow limits (± 0.2%) in the human body. Water balance is achieved by a balance between intake and output of water from the human body. Water balance is controlled through variable water loss from the urinary system, while
loss from the skin, the respiratory system and the gastrointestinal tract is under less regulatory control. Additionally, hormonal mechanisms regulating thirst play a significant role in maintaining water balance. Thirst is a physical manifestation of water imbalance, stimulated by an increase in plasma osmolality, a decrease in plasma volume, or a decrease in blood pressure. The main responsibility of the hormone, arginine vasopressin (anti-diuretic hormone), is to regulate the body’s retention of water. This hormone is activated when the body is dehydrated and thus it acts upon the kidneys to conserve water (Bossingham, 2005). Water input from drinking water, water in beverages and water from food restores the fluids lost through the various excretory methods (D’Anci, 2006). Water imbalance affects the hydration status of the body. Dehydration is an imbalance in the water intake and output that has been associated with certain chronic diseases. Dehydration, which is the depletion of water from the body, may adversely contribute to various chronic diseases; one in particular is osteoporosis (IOM, 2004). Osteoporosis-related fractures account for 1.5 to 2 million fractures annually, with an estimated cost of $13.7 to 20.3 billion (Cranney et. al., 2007).
2.1.3 Hydration Status and Osteoporosis

Osteoporosis is defined as a condition characterized by a decrease in bone mass and density that leads to increased bone fragility (Ringe, 2004). The prevalence of osteoporosis increases with age. Osteoporosis is a multifactorial disease in which nutrition plays a vital role. The risk factors of osteoporosis are classified as modifiable, unmodifiable, and treatable (Figure 2.1). Osteoporosis is characterized by continued calcium deficiency that leads to negative calcium balance. In this physical state the body relies on calcium from breakdown of the skeletal bone, which provides a reserve supply of calcium during low calcium intake (IOM, 1997 and Nordin, 1997). Osteoporosis increases bone fragility and susceptibility to increase risk of fractures (IOM, 1997). Currently, there are no longitudinal research studies that have addressed the effect of dehydration on osteoporosis. However, short term research studies have been conducted on the role of dehydration and the alteration in bone mineralization. The findings suggest hydration status impacts body composition and bone mineral density in healthy individuals (IOM, 2004).
2.1.4 Bottled Water Consumption

In 2003, bottled water emerged as the second largest commercial beverage category by volume in the United States. In the subsequent five years, the consumption of bottled water continued to increase. This sector of the market was growing even more forcefully on a global scale but in the US, volume increase was unmatched (Decker, 2006). From 2002 to 2007 bottled water sales increased 49%. In 2005, total US sales of bottled water were estimated between $50 and $100 billion (Gleick, 2004). However, in the past two years, the double-digit growth seen previously has been reduced to single digit growth. In 2007, there was an 8% growth compared to the previous year of 17% growth. The slower growth is partially due increased environmental concerns about plastic bottles adding to landfill waste (Dairy Foods, 2008).
However, even with the aforementioned slight reduction in growth; bottled water continues to be a key component in the beverage sector. Moreover, in 2005 bottled water volume exceeded 7.5 billion gallons, a 10.7% advance over the previous year. These findings suggest that the typical American consumes an average of 26.1 gallons annually. The average consumption of bottled water surpasses any other beverage, except for carbonated soft drinks (CSDs). Even though CSDs continue to be the most consumed beverage by volume, the soft drink market has been struggling recently due to competition from the bottled water sector (Decker, 2006). From 2002-07, CSBs grew a mere 5%, while non-carbonated beverages (milk, fruit juices, and functional beverages) increased 30%. This increase can be attributed to the heightened concerns about health (Dairy Foods, 2008). As non-carbonated beverages become more than a commodity purchase, industries are trying to expand this market sector by adding vitamins, minerals, sweeteners, flavor, etc. These line extensions have found niche markets that have increased sales in this beverage segment. Consumers’ perceive that these new products are a healthier replacement for traditional CSDs (Decker, 2003).

2.1.5 Flavored Fortified (Functional) Water Beverages

Flavored water has a stronghold in the beverage industry. Moreover, the fortified (functional) water beverage category is leading the industry. Functional beverages are defined as a non-alcoholic drink that administers health benefits apart from energy and essential nutrients. Vitamin waters and electrolyte-enhanced waters, both functional beverages, are competing within the beverage industry (Dairy Foods, 2008). Consumers have a growing desire to find a beverage that not only refreshes and has an acceptable taste but also offers a functional benefit. Furthermore, medical endorsement of healthy lifestyles including exercise, proper nutrition, and
overall wellness has contributed to the proliferation of this sector. Functional waters that have been fortified with vitamins and minerals are becoming increasingly accepted by consumers. Functional beverages are projected to increase from a $10 billion in 2004, to a $12.8 billion industry by 2009 (Decker, 2006).

2.1.6 Benefits

Popkin et al., (2006) recently proposed guidelines for beverage consumption after completing an in-depth review of research investigating effects of fortified beverages on health. The authors stressed the importance of a healthy diet including adequate intake of water for metabolism and normal physiological function (Popkin et al., 2006). The overwhelming evidence that current American diet is inadequate in good quality nutrition and fluid water fuels the flame that functional beverages have the ability to fill in some nutritional gaps (Kleiner, 1999 and Popkin et al., 2006). Functional foods, foods or food ingredients, have the ability to prevent or reduce the development of symptoms or diseases associated with nutritional inadequacies (Milner, 2000). Functional food formulation has progressed to the development and implementation of dietary supplements. For example, a current functional food utilized is probiotic and prebiotics in dairy products that aid in maintaining intestinal health (Charalampopoulos et al., 2002). The primary benefits of consuming functional water beverages are increased palatability, better hydration, physiological maintenance, and access to hydration. Continuous development of functional aqueous beverages may not only increase the beverage market but may play an important role in nutritional health (Decker, 2003).
2.2 History of Fortification

2.2.1 History

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

The United States continued to address the role of fortification as systematic approach to alleviate nutritional deficiencies. In addition, throughout World War II the enlistees frequently had poor nutritional status. To combat the issues of poor nutritional status and nutritional deficiencies, President Roosevelt assembled the National Nutrition Conference for Defense in 1941 which is currently referred to as the Food and Nutrition Board (FNB). A major result of this meeting was the recommendation for enrichment of flour and bread. Moreover, this coincided with the first presentation of the Recommended Daily Allowances (RDAs). RDAs were established for various vitamins and minerals and some of these components were approved as fortifiers. Thiamin, niacin, riboflavin, calcium, vitamin D and vitamin A were approved as fortifiers for such food products as flour, bread, milk and margarine. To be utilized as a fortifier a given nutrient must reflect standards established by the FNB and the Council on Foods and Nutrition of the American Medical Association (AMA):
The intake of the nutrient, in the absence of fortification, is below the desirable level in diets of a significant number of people.

The food from which the nutrient is to be derived is likely to be consumed in quantities that will make a significant contribution to the diet of the population in need.

The addition of the nutrient is unlikely to create an imbalance of essential nutrients.

The nutrient added is stable under proper conditions of storage and use.

The nutrient is physiologically available from the food to which it will be added.

There is a reasonable assurance against intake sufficiently in excess to be toxic (IOM, 2003).

2.3 Vitamin D

2.3.1 Calcium Connection

An intimate relationship exists between vitamin D and calcium. The synergistic interactions between calcium and vitamin D have been well documented (Iwamoto et al., 2004). Adequate amounts of calcium and vitamin D throughout life helps reduce the risk of abnormal bone formation, osteoporosis, high blood pressure, obesity and colon cancer (Calvo et al., 2005). However, to truly understand the biological functions of vitamin D, an investigation of the evolutionary relationship between the above aforementioned vitamin and mineral must be addressed.

The relationship between calcium and vitamin D began over 400 million years ago. At this time period there were environmental changes that caused many ocean-dwelling vertebrates to venture onto land. Previously, these vertebrates dwelled in an ocean environment that provided significant amounts of calcium for signal transduction as a pivotal regulator of a wide
variety of cellular metabolic events. However, when the vertebrates transitioned to land the new environment was insufficient in calcium. Therefore an adaptation occurred among the vertebrate species to allow for utilization of sunlight and synthesis of vitamin D to regulate intestinal absorption of calcium. This transformation allowed better usage calcium from the diet and had a positive impact on skeletal mineralization (Holick, 1999).

The role of calcium in mammalian species is well understood. Calcium is a major constituent of mammalian mineralization. Calcium aids in the normal growth and development of bone (Bronner, 1998). The majority of calcium (~99%), an essential nutrient, is found in the bones and teeth. The remaining 1% is contained in the serum, extravascular fluid, muscle, and other tissues (Committee of Nutrition, 1999). This nutrient is regulated by several hormones including the Parathyroid Hormone (PTH), calcitonin, and vitamin D. These regulators, when operating appropriately, maintain calcium balance within narrow limits. A low intake of calcium stimulates the breakdown of bone to supply the needed calcium for normal physiological functions. Continued release of calcium from the bone causes a reduction in bone mass and increases the risk of osteoporosis. Good sources of calcium are abundant throughout the U.S. Calcium can be obtained from calcium rich foods such as milk, dairy products, and other fortified foods such as cereal and juice beverages (IOM, 1997). The Institute of Medicine (IOM) recommended adequate intake of calcium for adolescents and adults ranges from 1200- 1300 mg/day (IOM, 1997).
Table 2.1 Recommended Adequate Intake by the IOM for Calcium

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

(Institute of Medicine, 1997)

Calcium is transported by active transport and passive diffusion. Active transport is defined as the movement of calcium, entry, intracellular diffusion, and extrusion which requires metabolic energy. Active transport is the primary mechanism of calcium absorption at low and moderate intake and is highly regulated by 1, 25 dihydroxyvitamin D, the active form of vitamin D. Conversely, passive diffusion is imperative with high intakes of calcium diffusion across the intestinal mucosa (Bronner, 1998; IOM, 1997). Calcium is absorbed in the small intestine (duodenum) with favorable low pH (<6) (IOM, 1997).

2.3.2 Source, Structure, and Synthesis

Previously little was known about the evolutionary perspective of vitamin D (calciferol). In 1919, a scientist named Edward Mellanby discovered the importance of vitamin D on development, growth and maintenance of a healthy skeletal system when investigating the prevalence of rickets (Holick, 2003). In addition, throughout the years of 1924 - 1927 researchers investigated vitamin D found in oily fish and in fish liver oils. It was believed that the vitamin D in these components were due to dietary intake of vitamin D from phytoplankton.
and zooplankton. These scientists were able to demonstrate that seasonal variation in vitamin D could be noticed in oily fish and fish liver, with the highest amounts of vitamin D during the summer months and a lower content of vitamin D during the winter months (Holick, 2003, Hollis, 2005).

As illustrated in Figure 1.2, vitamin D comes in various forms, but the two most prominent forms are ergocalciferol (vitamin D₂) and cholecalciferol (vitamin D₃) (IOM, 1997). Vitamin D₂ is derived from yeast and plant sterol, and is the usual form of vitamin D employed during fortification. On the other hand, vitamin D₃ is obtained when radiant energy (UV light) from the sun interacts with 7-dehydrocholesterol, a precursor of cholesterol, found in the skin. During exposure to sunlight, provitamin D is thermally isomerized to vitamin D and translocated throughout the body via vitamin D binding protein. Sufficient sun exposure can generate adequate active vitamin D for biological functions. However, seasonal, geographical latitude, time of day, cloud cover, environmental smog, skin melanin content (increased pigmentation), and use of sunscreen to prevent development of skin cancer are among some of the factors that affect vitamin D synthesis via UV radiation exposure. Due to the fact that the body is capable of producing cholecalciferol, vitamin D is not defined as the classical vitamin. A more accurate description of vitamin D is a calcitropic prohormone; which indicates that the vitamin is metabolized to a biologically active form that functions as a steroid hormone. Nonetheless, since the importance of vitamin D has been discovered it has been classified among the lipid-soluble vitamins (IOM, 1997, Lipkin, 2006). The primary sources of vitamin D in nature are very few foods including fish liver oils (manufactured through ultraviolet irradiation), the flesh of fatty fish (manufactured through ultraviolet irradiation), and eggs from
hens fed vitamin D enriched diet (vertical transfer) (Holick, 2007). Fortified food products that provide vitamin D include milk, margarine, breads and fortified breakfast cereals. Figure 1.2, illustrates food sources of vitamin D and amount of vitamin D contained in each source. Throughout the world the major source of vitamin D is via exposure to sunlight (Hollis, 2005 and IOM, 1997). Researchers have shown that D$_2$ and D$_3$ have the same biological potency in humans (Mata-Granados et al., 2009). Conversely, in the vitamin D research community there is debate over which vitamer (D$_2$ vs. D$_3$) is more bioavailable. Of the few comparative studies, vitamin D$_3$ has been seen as more bioavailable, thus the majority of vitamin D fortified foods in the United States is with D$_3$ (Armas et al., 2004, Trang et al. 1998).
Table 2.2 Selected Food Sources of Vitamin D

<table>
<thead>
<tr>
<th>Food</th>
<th>IUs per serving*</th>
<th>Percent DV**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cod liver oil, 1 tablespoon</td>
<td>1,360</td>
<td>340</td>
</tr>
<tr>
<td>Salmon, cooked, 3.5 ounces</td>
<td>360</td>
<td>90</td>
</tr>
<tr>
<td>Mackerel, cooked, 3.5 ounces</td>
<td>345</td>
<td>90</td>
</tr>
<tr>
<td>Tuna fish, canned in oil, 3 ounces</td>
<td>200</td>
<td>50</td>
</tr>
<tr>
<td>Sardines, canned in oil, drained, 1.75 ounces</td>
<td>250</td>
<td>70</td>
</tr>
<tr>
<td>Milk, nonfat, reduced fat, and whole, vitamin D-fortified, 1 cup</td>
<td>98</td>
<td>25</td>
</tr>
<tr>
<td>Margarine, fortified, 1 tablespoon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ready-to-eat cereal, fortified with 10% of the DV for vitamin D, 0.75-1 cup (more heavily fortified cereals might provide more of the DV)</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>Egg, 1 whole (vitamin D is found in yolk)</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td>Liver, beef, cooked, 3.5 ounces</td>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td>Cheese, Swiss, 1 ounce</td>
<td>12</td>
<td>4</td>
</tr>
</tbody>
</table>

*(ODS, 2008)*

*IUs = International Units.*

**DV = Daily Value. DV’s were developed by the U.S. Food and Drug Administration to help consumers compare the nutrient contents of products within the context of a total diet. The DV for vitamin D is 400 IU for adults and children age 4 and older. Food labels, however, are not required to list vitamin D content unless a food has been fortified with this nutrient. Foods providing 20% or more of the DV are considered to be high sources of a nutrient.
2.3.3 Absorption and Digestion

Enzymes in the liver and kidney convert the prohormone form of vitamin D to the active form of vitamin D (Figure 1.3). When adequate amounts of vitamin D are ingested via food, approximately 80% vitamin D is incorporated into the micelles of the small intestine and absorbed along with the dietary fat in the intestine. Exposure to sunlight converts 7-dehydrocholestrol to vitamin D. Vitamin D binds to the protein carrier in the blood and is transported to the liver by chylomicrons via the lymphatic system. While in the liver, vitamin D is hydroxylated to 25-OH vitamin D, the circulating form, and further metabolized to the active
hormone form 1, 25 dihydroxy vitamin D by the kidney; the active vitamin D is transported to the target tissue in the body. It has been determined that tissues other than the kidney have the ability to produce 1,25 dihydroxyl vitamin D. Many tissues in the body, including the prostate, colon, breast, skin, osteoblasts have the ability to express 1-α-hydroxylase and have the ability to synthesis 1,25(OH)₂ D₃ (Tangpricha, 2001). The aforementioned process, may be causal event for the prevention of development of certain chronic diseases. Moreover, the metabolism of vitamin D is feasible due to a specific protein carrier. Vitamin D that is circulated throughout the body is bound to a specific protein carrier, vitamin D binding protein (DBP) (Rowling, 2006). DBP binds the metabolites of vitamin D with different affinities: 25(OH)D > 24,25(OH)₂D > 1, 25(OH)₂D > vitamin D. This binding capacity facilitates the delivery of vitamin D to various sites for metabolism, storage and action (Dusso, 2005). Vitamin D is stored in the liver and adipose tissue. Excretion of vitamin D metabolites occurs via the bile, with a minute amount via the urine (Holick, 2003).
2.3.4 Function

After vitamin D has been metabolized into its active form it can contribute to the biological activity of the body. Vitamin D₃ plays a significant role in the regulation of blood calcium by increasing absorption via the small intestine, reducing calcium excretion via the kidney and regulating calcium loss from the bones. The parathyroid hormone (PTH) regulates the production of 1,25-(OH)₂D₃ when calcium blood levels are low (IOM, 1997).
Moreover, vitamin D plays a significant role in the maintenance of serum calcium homeostasis and phosphorus homeostasis within the range that supports normal neuromuscular function, bone calcification and other cellular processes. Additionally, calcium and phosphorus work synergistically to promote normal bone mineralization. Maintenance of calcium and phosphorus within narrow limits by vitamin D is very important for all living organisms (IOM, 1997).

2.3.5 Effect of Vitamin D Deficiency

Historically, rickets, first discovered in 1645, was of profound concern at the turn of the 20th century in northern Europe and North America (Eitenmiller et al., 2008). Industrialization of many cities during this period provided an environment that obscured sunlight, which decreased the photosynthesis of vitamin D in the skin. The lack of exposure to sunlight limited skin’s production of vitamin D and the low availability of naturally vitamin D containing foods led to suboptimal vitamin D levels within the body. Low levels of vitamin D throughout the bloodstream have been associated with low or low-normal blood levels of calcium and phosphorous. Inadequate vitamin D in the body elevates serum parathyroid hormone (PTH), which facilities an increase in bone resorption. The condition of prolonged insufficient vitamin D is termed rickets in children, which was first described in 1645, and osteomalacia in the adult population. These conditions are common in children and women who have inadequate amount of vitamin D in the body (Holick, 2005).

Currently, vitamin D deficiency is still of global concern. Everyone is at risk of vitamin D deficiency due to many confounding factors (Holick, 2005; Jasinghe, 2006). Research studies have estimated that 90% of circulating serum vitamin D is due to sunlight exposure (Lapp,
However, researchers have reported that the majority of US citizens are exposed to suboptimal levels of sunlight, mainly during the winter months (Holick, 1992). For optimal conversion of 7-dehydrocholesterol to vitamin D, UVB irradiation from sun exposure of 20 mJ/cm² is required. In the northern United States above latitude 40°, sun exposure levels do not meet the requirements of 20 mJ/cm² (Hollis, 2005). Rickets and osteomalacia may occur in breastfed infants who do not receive vitamin D supplementation, women who have inadequate vitamin D intake from food and/or lack of exposure to ultraviolet light for photosynthesis of vitamin D, (Vieth, 2001) and older individuals with age-related synthesis of vitamin D (Eitenmiller, 2008).

2.3.6 Effect of Vitamin D Insufficiency

Recommended nutrient intakes are intended to prevent the development of nutrient deficiencies and insufficiencies (Yates, 1998). Current vitamin D recommendations have been shown to prevent vitamin D deficiency as it relates to the traditional bone and teeth health. However, the identification of other non-calcitropic autocrine and paracrine functions of vitamin D has led the scientific community to reassess current vitamin D recommendations. These suggestions have led to the additional classification of vitamin D ‘insufficiency’ (Cranney et al., 2007). Vitamin D insufficiency is a condition when serum 25-hydroxyvitamin concentrations are within the normal ranges (30 -74.0 nmol/L), but the values are at the lower half of the normal range (Vieth et al., 2001). Vitamin D insufficiency has an inverse biological relationship between serum 25(OH)D levels and parathyroid hormone. There is an excess of parathyroid hormone associated with vitamin D insufficiency, which may promote mineral loss. The mineral loss may cause osteoporosis or development of other chronic diseases (Chapuy et
Numerous cross-sectional research studies have found a direct link between vitamin D insufficiency and increased risk of developing cancer, cardiovascular disease, diabetes mellitus, hip fractures, and osteoporosis (Calvo et al., 2005). As stated earlier, vitamin D and calcium work together synergistically. However, calcium may not be needed for vitamin D to work with VDR-RXR (retinoic acid receptor) molecules to regulate cellular proliferation, differentiation, and function. When the above relationship does not exist, vitamin D causes cellular dysfunction and increase risk of developing certain chronic disease (Peterlik et al., 2009).

2.3.7 Vitamin D Fortification

In 1941, President Roosevelt issued a request for fortification of food products with vitamin D. His goal was to ensure that consumers had a sufficient amount of vitamin D in the diet to alleviate the epidemic of vitamin D deficiency in industrialized communities. This outcry from President Roosevelt resulted in vitamin D-fortified milk. Currently, there are many other food items fortified with vitamin D including yogurts, cereals, orange juice, and nutritional bars. However, with additional fortification of certain food products with vitamin D, research studies continuously report that the current fortification is inadequate or not found in enough different food products to prevent vitamin D deficiency (IOM, 2003).

Due to the concern of adequate vitamin D intake and challenges associated with vitamin D fortification beyond the traditional media, it is apparent that research must address these issues. One study in particular, conducted by Johnson et al. (2005) investigated the bioavailability of vitamin D from fortified processed cheese. This two part study, first determined the effect of two months of daily consumption of vitamin D (600 IU) fortified cheese on serum levels among the elderly population. These results illustrated no significant
difference in serum levels among those who consumed the fortified processed cheese. These findings lead to further investigation of vitamin D bioavailability in different foodstuff. The second part of the study compared bioavailability of fortified cheese (5880 IU D2/56.7g) versus water (containing 32,750 IU/250 mL) among different age groups (older versus younger) subjects who received an acute amount of vitamin D on serum vitamin D levels. These results showed that serum levels were significantly lower with consumption of fortified water than with fortified processed cheese. In addition, the peak serum vitamin D levels were similar among both age groups. Moreover, the acute amount of vitamin D in study two was indeed bioavailable when compared to the processed cheese consumed in study 1, which had no significant effect on blood serum levels. This study also highlighted the primary concern in the fortification of foods which is recovery of vitamin D after processing by various methods. In conclusion, research studies must continue to investigate vitamin D fortification techniques and quantification in various food sources (Johnson et al., 2005).

Table 2.3 – 1997 RDI Adequate Intakes for Vitamin D

<table>
<thead>
<tr>
<th>RDI for Vitamin D</th>
<th>µg/day (5µg = 200IU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Babies</td>
<td></td>
</tr>
<tr>
<td>Birth - 12 months</td>
<td>5</td>
</tr>
<tr>
<td>Children</td>
<td></td>
</tr>
<tr>
<td>1-13 years</td>
<td>5</td>
</tr>
<tr>
<td>Adults</td>
<td></td>
</tr>
<tr>
<td>14-50 years</td>
<td>5</td>
</tr>
<tr>
<td>51 - 70 years</td>
<td>10</td>
</tr>
<tr>
<td>Over 70 years</td>
<td>15</td>
</tr>
<tr>
<td>Pregnant Women</td>
<td>5</td>
</tr>
<tr>
<td>Nursing Women</td>
<td>5</td>
</tr>
</tbody>
</table>

(Institute of Medicine, 2003)
2.4 Vitamin D Receptor (VDR)

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

VDR, a nuclear receptor for 1α,25(OH)₂D₃, the active form of vitamin D₃, plays a critical role in bone formation. VDR is responsible for activation of calbindin, a calcium transport protein in the small intestine. Moreover, the distribution of VDR throughout the body demonstrates the various functions of 1α,25(OH)₂D₃ beyond mineral and skeletal homeostasis (Holick, 2003 and Veith 2001). Vitamin D₃ is responsible for cell proliferation and inhibition or maturation of normal and tumor cells. Tanaka et al. (1982) investigated the effect of vitamin D on preleukemic cells in rats that were vitamin D sufficient. The researchers found that 1α,25(OH)₂D₃ and VDR work synergistically to inhibit growth and differentiation of cancerous cells (Holick, 2003). Vitamin D halts cancer growth by working through nuclear receptors that regulate gene expression and protein synthesis. The VDR binds 1α,25(OH)₂D₃ in the cytosol then has to bind to RXR(retinoic acid receptor) to get the nucleus to activate or deactivate appropriate genes (Deeb et al., 2007).
2.5 Beta-lactoglobulin (BLG)

Beta-lactoglobulin (β-LG), a small soluble protein that is extremely acid stable, is the major whey protein present in milk (Table 1.4). Whey protein is one of the highest quality commercially available proteins (Perez, 1990). Whey protein is comprised of a higher concentration of branched-chain amino acids (BCAA) and essential amino acids than most other protein sources. In addition, other components of whey, such as β-LG and peptides have been shown to promote increased protein synthesis, weight loss, body fat loss, and decreased plasma insulin and triglyceride profile. Therefore, the protein components of whey have been continuously researched for the nutritional, nutraceutical, and health benefits (Onwulata et al., 2008).
**Table 2.4 Composition of Whey Protein in Bovine Milk**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Approximate % of Whey Protein</th>
<th>Benefits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta-lactoglobulin</td>
<td>50-55%</td>
<td>Source of essential and branched chain amino acids</td>
</tr>
<tr>
<td>Alpha-lactalbumin</td>
<td>20-25%</td>
<td>Primary protein found in human breast milk. Source of essential and branched chain amino acids</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td>10-15%</td>
<td>Primary protein found in colostrum immune modulating benefits</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>5-10%</td>
<td>Source of essential amino acids</td>
</tr>
<tr>
<td>Glycomacropeptide (GMP)</td>
<td>2-5%</td>
<td>Source of branched chain amino acids</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lacks the aromatic amino acids phenylalanine, tryptophan, and tyrosine</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>1-2%</td>
<td>Antioxidant</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antibacterial, antiviral, and antifungal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Promotes growth of beneficial bacteria</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Naturally occurs in breast milk, tears, saliva, bile, blood, and mucus</td>
</tr>
</tbody>
</table>

(Marshall, 2004 and Onwulata et al., 2008)

### 2.5.1 Source, Structure, and Synthesis

Beta-lactoglobulin production occurs in the mammary gland with inclusion in milk. $\beta$-LG accounts for approximately 10% of total milk protein or 55% of total whey protein. It was discovered in 1934 by Palmer during the salt fractionation of the whey fraction of cow’s milk.
This purification of β-lactoglobulin by Palmer has provided the framework for investigating the physico-chemical properties and potential uses of this protein (Sawyer et al., 1999).

Although it was previously believed that β-LG was only found naturally in ruminant species, the protein is present in the milk of most mammalian species, excluding rodents and primates. Therefore, β-LG has been characterized in most detail from ruminants. This model has provided insight into the nature of the protein. Within this order, β-LG exists as a dimer of subunit with a relative molecular weight approximately 18,400 daltons for generic variant A and 18,276 daltons for B (Papiz et al., 1986). Each monomer contains 162 amino acids, with one free cysteine and two disulphide bridges. The complete amino acid sequence of β-lactoglobulin genetic variants A and B differ in two amino acid residues – Asp 64 and Val 118 in variant A are replaced in variant B with Gly and Ala (Wong et al., 1996). The crystalline structure of β-LG is similar to retinol-binding protein and other proteins in the lipocalin family, which are generally noted to transport small hydrophobic molecules (Kontopidis, et al., 2002). The configuration of the protein is a β-barrel with eight antiparallel β-strands to form a conical central cavity in which the hydrophobic ligand is located. On the outer surface of the β-barrel there is a three-turn α-helix. The secondary structure of the protein is 15% α-helix, 50% β-sheet, and 15-20% of reverse turn. This configuration plays a significant role in the binding ability of β-lactoglobulin (Wu et al., 1999).

As noted earlier, the abundant knowledge pertaining to β-LG has been ascertained from ruminant animals. Moreover, β-LG is acid stable, and resistant to denaturation at pH as low as pH 2.0. Bovine β-LG is resistant to gastric digestion in vivo and remains intact when passing
through the stomach. The protein is most heat sensitive near pH 4.0, with the maximum stability at pH 6.0 and decreasing stability in the higher pH range (Resch, 2004).

2.5.2 Beta-lactoglobulin Interaction with Vitamin D

β-Lactoglobulin has been shown to have the ability to bind in vitro to certain hydrophobic molecules such as retinoids, retinyl palmitate, vitamin D, cholesterol and some other fatty acids. The β-lactoglobulin molecule has been shown to function in binding and/or transport of small hydrophobic molecules (Kontopodis et al., 2004). Research studies have investigated the binding ability of vitamin D to β-LG, and found that vitamin D can bind to β-LG. The β-LG molecule structurally has two hydrophobic pockets that are capable of binding lipopholic molecules. The sites of binding are the calyx formed by the β-barrel and the other between the α-helix and the surface of the barrel. The binding site of lipopholic molecules is still controversial, but most scientific research finds vitamin D binds near the monomer contact surface of the dimer. Fluorescence spectroscopy has denoted a change in tryptophanyl fluorescence, which indicates the binding of lipopholic molecules to β-LG. Therefore, the binding of these two molecules will produce a complex that may be stable and soluble in aqueous solutions (Wang et al., 1997). This complex may be ideal for the fortification of an aqueous bottled beverage (Kontopodis et al., 2004).
Figure 2.5- Structure of Beta-Lactoglobulin
(www.Bio-Rad.com)
2.6 High-Performance Lipid Chromatography

High-performance lipid chromatography (HPLC) is a predominant method for separation and quantification of various compounds including pharmaceuticals, biomolecules, polymers, and other organic and ionic compounds (Dong, 2006). HPLC is divided into several types however the two most common are normal-phase chromatography (NPC) and reverse-phase chromatography (RPC). Normal phase separates analytes based on adsorption/desorption (typically silica or silica modified with CN and NH₂ groups) and polarity (typically silica or alumina). The mobile phase utilizes non-polar solvents hexane with addition of small amount of more polar solvent including iso-propanol, ethanol or chloroform. This type of chromatography is particularly useful for separation of small non-polar and cis/trans compounds. A main disadvantage of normal phase chromatography is the ease of contamination of the instrument’s column by sample components. Whereas RPC is the most common separation mode, accounting for approximately 70% of all HPLC analyses. RPC separates based on a polar mobile phase and a hydrophobic (non-polar) stationary phase. It is used in many pharmaceutical, biochemical, and analytical chemistry applications (Dong, 2006). Separation occurs primarily because of solvophobic or hydrophobic interaction. The non-polar column is typically made from C18 or C8 material, and a more polar molecule. RPC is advantageous to the scientific community because it is the most stable HPLC method. Conversely, disadvantages for RPC include an increased time needed for analysis and re-equilibration of the column. Moreover, it is necessary to evaporate the extract to dryness and dissolve the residue in small amounts of methanol before performing the HPLC procedure. HPLC, with all its advantages...
and disadvantages (Table 2.5), continues to be the primary tool for quantitation of various samples including vitamin D from most matrices (Mata-Granados, et al., 2009).

Research has shown that both NPC and RPC can be utilized to quantify vitamin D in solutions. The two official methods for vitamin D determination are AOAC international official method 981.17 for fortified milk and milk powder and AOAC international official method 995.05 for infant formulas and enteral products. Both of these methods rely on normal-phase HPLC to determine total vitamin D in a system, but neither allow for the vitamers to be used as an internal standard. RPC is more readily employed because it has the ability to separate the vitamers (i.e. D<sub>2</sub> vs. D<sub>3</sub>) and allow use of an internal standard. NPC is unable to identify the amount of D<sub>2</sub> compared to D<sub>3</sub>. However this procedure is able to separate vitamin D, 25-hydroxyvitamin D<sub>2</sub>, and 25-hydroxyvitamin D<sub>3</sub> and other hydroxylated metabolites (Perales et al., 2005). Taking the factors of this discussion into account RPC, was the method utilized for this research.

**Table 2.5 Advantages and Limitations of HPLC**

<table>
<thead>
<tr>
<th>Advantages:</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Rapid and precise quantitative analysis</td>
</tr>
<tr>
<td>• Automated operation</td>
</tr>
<tr>
<td>• High-sensitivity detection</td>
</tr>
<tr>
<td>• Quantitative sample recovery</td>
</tr>
<tr>
<td>• Amenable to diverse samples</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Limitations:</th>
</tr>
</thead>
<tbody>
<tr>
<td>• No universal detector</td>
</tr>
<tr>
<td>• Less separation efficiency than capillary GC</td>
</tr>
<tr>
<td>• More difficult for novices</td>
</tr>
</tbody>
</table>

(Dong, 2006)
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CHAPTER 3

Preparation of Protein-Vitamin Complex Beverages

Mallorye D. Lovett

Department of Food, Bioprocessing and Nutrition Sciences
North Carolina State University
ABSTRACT

Beta-lactoglobulin (β-LG) is a major component of whey protein. It is a member of the lipocalin family, and has been reported capable of binding hydrophobic compounds. β-LG has the ability to bind retinol (vitamin A), fatty acids, cholesterol, and vitamin D. This binding ability has shown importance because β-LG could serve as a carrier of hydrophobic compounds, more specifically vitamin D, without the presence of the fat in which they are normally associated. In order to justify the potential of β-LG as a transporter, stabilizer, and protective carrier for vitamin D, stability of β-lactoglobulin complexed vitamin D in an aqueous solution was investigated.

In this study, a β-LG vitamin D complex was created via spray drying. The process of spray drying has the potential to denature the protein, which may result in low retention of vitamin D due to exposure to high temperature (heat) during the procedure. In addition, certain sugars such lactose and trehalose, have been found to exhibit characteristics that allow for the stabilization of whey protein during spray drying. The objective of this research was to observe the stability of β-LG vitamin D complex in an aqueous solution with the use of either lactose or trehalose. The stability of the complex was observed by High Performance Liquid Chromatography (HPLC) and an Enzyme-Linked Immunosorbent Assay (ELISA) based procedure. Results showed that there was no difference in binding when comparing different sugars. Additionally, β-LG vitamin D complex remained stable in an aqueous solution.

In conclusion, β-LG vitamin D complex is an appropriate fortifier for an aqueous solution and β-LG has the ability to protect and transport vitamin D.
3.1 Chapter Overview

As a member of the lipocalins family, beta-lactoglobulin (BLG), like bovine serum albumin, has many functional properties including binding and transportation of many hydrophobic molecules such as vitamins. β-LG’s structure has the ability to tightly bind to retinol (vitamin A), cholesterol and vitamin D (Kontopidis, 2004). The aforementioned properties have continued to fuel research and the interest in use of β-LG in the food industry.

The spray drying process is a commonly used method for dehydration of fluids. This process has been applicable with processing of milk, whey, buttermilk, butter, ice cream mixes, and other dairy products. Food and pharmaceutical industries are investigating spray-drying as a means of incorporating vitamins and medication into various application systems (Ameri, 2006). However, with these applications the heat from the spray drying equipment is of concern. The structure and solubility of β-LG and other whey proteins are interrelated and affected by most heat treatments including spray drying. Heat can lead to denaturation of the β-lactoglobulin protein and cause a complex to dissociate. In order to produce a β-LG–vitamin complex, heat must be controlled to prevent denaturation (DeWitt et al., 1984). Even though, denaturation of β-LG may be a disadvantage (control point) during processing there are many advantageous reasons to utilize spray drying as a mechanism to increase functionality in dairy products, pharmaceutical agents, and other processing avenues.

Therefore, the purpose of the study was to spray-dry a solution containing a complex of β-LG and vitamin D to produce a powder that was stable and could conveniently fortify non-fat food or aqueous solution. The information gathered was used to create a model vitamin D fortifier that could be used in a human bioavailability intervention. The chapter then describes
experiments to demonstrate binding of the particular “water soluble” form of vitamin D used in this study to β-LG, and a shelf-life study to demonstrate the stability of the vitamin-D fortified beverage in comparison with vitamin-D milk.

3.2 Spray-drying of β-LG-vitamin complexes

3.2.1 Introduction

Although many drying methods are available, spray drying is one of the most abundantly used drying methods in both the food and pharmaceutical industries. The method of spray drying has been used since the 1950s in procedures such as encapsulation of flavor oils to protect against degradation and oxidation, preservation of vitamins, and the traditional conversion of a liquid to a powder (Desai et al., 2009). Typically this method results in a dry powder from a liquid, suspension, or emulsion by rapidly drying a given solution with hot gas pumped through an atomizer (Tratnig et al., 2009). The atomizer is a rotating wheel or nozzle that disperses the liquid into a high-velocity stream of dry hot air, which results in the production of droplets (Desai et al., 2006). The spray dryer takes a liquid stream and separates the solution into solute phase as a suspension that forms droplets and the solvent into vapor phase. The droplets formed pass through the controlled heated atmosphere by either co-current or counter-current flow, while simultaneously rapidly evaporating the moisture. Solid material is formed as the liquid is quickly vaporized, and the large particles fall to the bottom of the chamber and are collected. The fine particles entrapped with the hot gases go through cyclone and are collected in external cyclones or HEPA or baghouse filters (Garcia-Munoz et al., 2009). Moreover, the role of the atomizer is to produce small droplets ranging from 20 to 180 μm because the droplet dimension plays a critical role in heat transfer and the rate of water
vaporization. These conditions create a protective film around the droplets so that the particle is kept at the saturation temperature. As a result of this phenomenon, heat-sensitive products, such as beta-lactoglobulin and vitamins can be spray dried at relatively high air temperature if the solids do not approach or exceed the dryer outlet temperature (DeWitt et al., 1984).

Sugars such as lactose and trehalose have been shown to play an important role in the stabilization of the β-LG conformation when drying whey protein. Sucrose and trehalose are both disaccharides and have been shown to stabilize protein structure during processing (Richards et al., 2002 and Liao et al., 2002). Studies have shown that trehalose may play an even greater role in maintaining the conformation of the protein and increase binding of the ligand (Liao et al., 2002). The purposes of this experiment were a) determine the effectiveness of lactose verses trehalose in preserving the β-LG configuration, that is favorable for vitamin D binding; b) to spray-dry the β-LG –vitamin complex solutions and produce stable and convenient fortifiers of vitamins for a brief storage study and c) compare the accuracy of the traditional HPLC vitamin analysis with a new ELISA.

3.2.2 Material and Methods

3.2.2.1 β-lactoglobulin and Vitamin D Complex Preparation

BioPure β-lactoglobulin was a gift from Davisco Foods International, Inc. (Le Sueur, MN). Vitamin D₃ powder (MW 384.65) and vitamin D₂ powder (MW 396.65), and lactose were purchased from Sigma Chemical Company (St. Louis, MO) and Roche Vitamins Inc. (Nutley, NJ), respectively. Additionally, food grade vitamin D₃ (D₃’Sol) liquid and vitamin D₂ (D’Sol) liquid were supplied by Freeman Industries, LLC (Tuckahoe, NY). Star Vitamin D₂ (Danisco, Copenhagen, Denmark) and Vitamin D₃ (Pat Vitamins, City of Industry, CA), both food grade
reagents, were also utilized in analysis. Trehalose was provided by Hayashibara International Inc., (Broomfield, CO). Four hundred mL of 2% (w/v) of β-lactoglobulin (~8 g) in DI water was prepared. The solution was mixed on a magnetic stirring plate at low speed (2) to prevent foaming until a homogenous clear solution was obtained. This technique was employed to prevent the denaturation of β-LG. When β-LG is exposed to high speeds there is an increase in protein breakdown and also reduction in the binding capacity of β-LG to vitamin D. Cholecalciferol (D₃) and Ergocalciferol (D₂) in the amount of 0.18 g were dissolved by 400 µL absolute ethanol, the above solutions were pipetted into the beta-lactoglobulin solution until 1:1 molar ratio of vitamin to monomer of protein was reached. The protein-vitamin solution was incubated at 40°C for 2 hours according to the method used by Kontopidis et al. (2004). For the complex with lactose and trehalose, 5:1 weight ratio of lactose/trehalose to protein was added to the solution after the incubation and the mixture was allowed to sit at room temperature until a homogenous clear solution resulted. Work conducted with vitamin D was performed under dim yellow light and amber containers enclosed in aluminum foil to prevent degradation of vitamin D from direct light exposure.

3.2.2.2 Spray Drying β-lactoglobulin and Vitamin D Complex

The complex was spray dried on a pilot scale dryer (Annhydro, Denmark). The equipment parameters of 120°C inlet air temperature and 68-70°C outlet temperature had to be achieved prior to utilization of the system. The system was flushed with deionized water via a Master Flex peristaltic pump (Model 7518-10, Cole-Parmer Instrument Co., Vernon Hills, IL) to stabilize the unit before the solution could be added. The protein-vitamin stock solution was
pumped into the machine at a flow rate ~2 mL/min while continuously observing the inlet and outlet temperatures to ensure that heat denaturation would not occur.

3.2.2.3 Powder Recovery

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

3.2.2.4 Vitamin D Analysis High Performance Liquid Chromatography (HPLC)

The vitamin D content of the β-lactoglobulin-vitamin D complex was determined by High Performance Liquid Chromatography (HPLC) (Figure 3.1) based on vitamin D₃ and vitamin D₂ as internal standards according to Liu (2003). HPLC analyses were performed on a Waters Millipore Automated Gradient Controller with UVIS linear detector and a manual loading injector (Waters Associates, Milford MA). Reversed-phase 4.6 x 250nm Vydc TP201 C18, 5 µm column with a guard column (Vydc, Hesperia, CA) was used with a mobile phase consisting of acetonitile/ethyl acetate/chloroform (88:8:4; v/v/v) at a consistent flow rate of 1 mL/min. Methanol was used to wash and equilibrate the column before and after each sample injection. A wavelength of 264 nm was used to quantify the results and Dynamax Software Package (Waters Associates) was used to integrate the peak areas of vitamin D. The flow parameters used during the analyses of the protein-vitamin complex are shown in Table 3.1.
3.2.2.5 Vitamin D Analysis VitaKit D™

The vitamin D content of beta-lactoglobulin–vitamin D complex was determined by VitaKit D™ (SciMed Laboratories Inc., Alberta, Canada). VitaKit D™ is a rapid ELISA, (Enzyme-Linked-Immunosorbernt Serologic Assay) which is based on the principle of a competitive binding enzyme immunoassay. The assay reduces analysis of vitamin D content from a 2-5-day procedure in typical HPLC analysis to a 2-3 hours procedure, by utilizing a fixed number of vitamin D₃ molecules immobilized on a solid phase. The unknown vitamin D molecules contained in milk samples or other liquids containing vitamin D compete for a fixed number of binding sites on the enzyme-labeled monoclonal antibodies. An inverse relationship exists between amount of vitamin D in the solution and the amount of enzyme – labeled antibodies contained within the assay (SciMed, 2007).

Both vitamin D powder samples and vitamin D liquid samples were prepared for evaluation according SciMed Laboratories Inc. protocol. There was variation in preparation of reagent between the powder and liquids. The preparation of powered samples will be discussed first. Powdered samples were brought to room temperature. One gram of thoroughly mixed powder was added to a 15-mL centrifuge tube. Distilled water was added until the final volume was 10 mL. The samples were vortexed to completely dissolve the powder. Once dissolved, the samples were incubated for 30 minutes at 45°C in a water bath. During the incubation period, preparation of 90% KOH was made (freshly made daily). The samples were inverted slowly approximately 20 times before continuing the extraction procedure. A sample (0.5 mL) of the reconstituted powdered samples was placed in a 10-mL labeled screw capped glass tube. Dimethy sulfoxide, (CH₃)₂SO, in the amount of 0.3 mL was added to each sample tube. The
tubes were placed on a shaker at 200 rpm for 2 minutes at room temperature. After completion of the shake period, 2 mL of 90% potassium hydroxide was added to each tube and samples were capped. The samples were returned to the shaker at 400 rpm for 2 minutes at room temperature. The samples then were incubated at 45°C for 10 minutes in a water bath. Samples were once again returned to the shaker at 400 rpm for 2 minutes, incubated an additional 10 minutes at 45°C in water bath, and followed by an additional incubation period of 10 minutes at room temperature. The samples were return to the shaker at 400 rpm for 2 minutes at room temperature. Once the samples were removed from the shaker, 2 mL of hexane was added to each test tube. The samples were then placed on the shaker at 400 rpm for 2 minutes at room temperature. The samples were then centrifuged at 2500 rpm for 5 minutes at room temperature. Two-hundred µL of the organic layer was removed and placed in a small labeled amber vial. The samples were tightly capped and the ELISA assay procedure immediately followed.

Liquid samples preparation procedure slightly differed from extraction of vitamin D from powdered samples. One mL of skim milk or non-fat containing vitamin D liquid samples was placed in a 10-mL screw capped glass tube. To each 1-mL sample, 0.3 g of KOH were added and gently mixed for two minutes at room temperature in the dark. The samples were then incubated for an additional 4 minutes in the dark and shaken vigorously for 2 minutes. This step was repeated three times. Two mL of hexane was added to the above solution and placed on the shaker for another two minutes in the dark. The tubes were centrifuged for 5 minutes. The upper organic phase was removed; 200 µL of vitamin-D extract was placed in
amber vials. The samples were tightly capped and the ELISA assay procedure immediately followed.

To analyze the vitamin D extract by ELISA, 10 µL of calibrators, extracted specimens, and controls were distributed into the corresponding wells of the 96-well microtiter plate. The wells were placed on the plate shaker (180 ± 10 rpm) for 8 minutes to evaporate the hexane. With a multi-channel pipette, 60 µL buffer solution was added and wells were mixed gently for 30 minutes. The wells were incubated for an additional 3 minutes in the dark. Sixty µL of anti-vitamin D conjugate was added to the wells before gently mixing for 20 seconds and incubating for 10 minutes in the dark. The wells were washed six times with 380 µL of distilled water. The excess moisture was removed by tapping the plate against an absorbent paper. Substrate was added to each well (60 µL) and gently mixed for 10 seconds and incubated for 5 minutes. To stop the competitive binding a stop solution was added to each well and the absorbance was read at 450 nm using a microplate reader.
3.2.2.6 Standard Curve and Recovery of Vitamin D from Complex

The standard curve for the VitaKit D™ was ascertained from internal standard solution samples contained within the kit. Standard curve formation for HPLC was as follows: Vitamin D...
D$_2$ and D$_3$ stock solutions were made by adding 0.02 g of vitamin powder with an additional 100 mL of methanol for a final concentration of 200 µg/mL. The concentrations of vitamin D$_2$ and D$_3$ were determined by a 1:100 dilution by obtaining the absorbency of the solution at 264 nm on a Genesys 2 spectrophotometer (Spectronic, Thermo Electron Scientific Instruments Co. Madison WI). The concentration calculations were determined by the following formula:

$$C = \frac{A}{E^o} \quad \text{or} \quad A = E^o BC$$

C = concentration

A = absorbance @ 264nm

E$^o$ = extinction coefficient; vitamin D$_2$ = 460; vitamin D$_3$ = 485

B = length (1cm)

Standard solutions were prepared by adding predetermined amounts of vitamin D to 4 mL amber vials according to Table 3.2. The vials were filled with 0.5 mL of methanol to re-dissolve the vitamin D. Fifty µL of each standard solution was injected to the HPLC system with the use of a syringe (Hamilton Co. Reno, Nevada microliter #805) for analysis.

**Table 3.2 Vitamin D$_3$ (Cholecalciferol) Standards**

<table>
<thead>
<tr>
<th>Vial #</th>
<th>Vitamin D3 working solution (2.5 µg/mL, 20 µL = 0.05 µg)</th>
<th>Vitamin D2 working solution (2.5 µg/mL, 60 µL = 0.15 µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>20 µL</td>
<td>60 µL</td>
</tr>
<tr>
<td>#2</td>
<td>40 µL</td>
<td>60 µL</td>
</tr>
<tr>
<td>#3</td>
<td>80 µL</td>
<td>60 µL</td>
</tr>
<tr>
<td>#4</td>
<td>120 µL</td>
<td>60 µL</td>
</tr>
<tr>
<td>#5</td>
<td>160 µL</td>
<td>60 µL</td>
</tr>
</tbody>
</table>
3.2.2.7 Assay of Vitamin D

3.2.2.7.1 Saponification

For HPLC analysis, a 0.02 g portion of vitamin D₃-fortified β-lactoglobulin powder was removed from the stock spray dried powder and placed in 45-mL screw-on top tube. Fifteen mL of DI water was added to the sample and spiked with 100 µL D₂ (internal sample). Fifteen mL of 1% ethanolic pyrogallol (made freshly daily) was added. The solution was cooled by flushing under nitrogen while adding ~6.0g of KOH pellets. Immediately the tubes were placed in ice to facilitate dissolution of the KOH pellets and control temperature of the samples. The tubes were wrapped with aluminum foil and shaken in darkness at room temperature overnight.

3.2.2.7.2 Extraction

After the completion to the saponification, the samples were transferred to 125-mL separatory funnels with 15 mL of DI water used to rinse the saponification tubes. An additional, 5 mL of ethanol and 45 mL of hexanes were added to the separatory funnels, the content was shaken for 1 minute and allowed to stand for 4 minutes. After the 4-minute time period, the aqueous layer (hexanes) was transferred to the Erlenmeyer flask and the organic layer was transferred to a 250-mL separatory funnel. The organic layer was washed two more times with 45 mL hexanes as described above. The pooled hexanes were placed in a 250-mL separatory funnel and 50 mL of 5% KOH was added. The mixture was shaken and allowed to stand for 4 minutes, and the aqueous solution was discarded. Fifty mL of DI water was added to the organic solution in a 250 mL separatory funnel, the mixture was shaken for one minute and allowed to stand for 4 minutes, the aqueous solution was discarded. An additional 50 mL of 55% ethanol:water was added to the organic solution contained in the separatory funnel, the mixture
was shaken and allowed to stand for 10 minutes. The organic layer was transferred to the previously collected hexane portion in a washed round bottom flask. The contents of the round bottom flask were placed on a Rotarvapor containing a water bath (Rotarvapor, BüCHI, Switzerland) to evaporate the hexanes at 40 to 50°C. After the round bottom flask was dry, 5 mL of hexanes was immediately added to the flask containing the vitamin D residue. The contents were transferred to centrifuge tubes. The round bottom flask was washed two more times with 3 mL hexanes, and contents were also collected and placed in test tubes. The solvent content of the centrifuge was removed under nitrogen flush. Once dried, the residue was re-dissolved in 500 µL methanol. The methanol residue mixture was filtered through 0.45-µm syringe filter into a 2-mL amber vial; samples were flushed with nitrogen and kept at -20°C until needed for further analysis.

### 3.2.2.7.3 Recovery of Vitamin D from β-LG Powder

Fifty µL of the extracted sample was injected into the HPLC system. Standards were injected before injecting the unknown samples. Area under the curve (AUC) was recorded for vitamin D2 and D3, respectively (Figure 3.2). The retention time was also obtained and recorded for the peak values under both vitamins (Table 3.3).
Figure 3.2  HPLC Chromatogram of Vitamin D

Table 3.3 Retention Time and AUC for Vitamin D$_2$ and D$_3$

<table>
<thead>
<tr>
<th>Sample</th>
<th>Retention Time</th>
<th>AUC in standard curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard 1</td>
<td>10.000</td>
<td>536537 D$_2$</td>
</tr>
<tr>
<td></td>
<td>11.700</td>
<td>1114001 D$_3$</td>
</tr>
<tr>
<td>1</td>
<td>10.033</td>
<td>358466 D$_2$</td>
</tr>
<tr>
<td></td>
<td>11.266</td>
<td>50772764 D$_3$</td>
</tr>
<tr>
<td>2</td>
<td>9.600</td>
<td>819134 D$_2$</td>
</tr>
<tr>
<td>1:10 dilution</td>
<td>11.100</td>
<td>15911161 D$_3$</td>
</tr>
<tr>
<td>3</td>
<td>9.450</td>
<td>330467 D$_2$</td>
</tr>
<tr>
<td>1:20 dilution</td>
<td>11.016</td>
<td>4629098 D$_3$</td>
</tr>
<tr>
<td>4</td>
<td>9.716</td>
<td>85395 D$_2$</td>
</tr>
<tr>
<td>1:40 dilution</td>
<td>11.216</td>
<td>1060833 D$_3$</td>
</tr>
</tbody>
</table>
3.2.3 Results

Spray-Drying of β-LG Vitamin Complexes

After spray drying, 36 g of protein was recovered from the original 50 g. The powder recovery rate was approximately 70% for each complex combination (trehalose or lactose). The recovered spray-dried material was analyzed by HPLC procedures. Prior to analyses of vitamin D content in the spray-dried β-LG vitamin complex, a standard vitamin D curve was developed, as illustrated by Figure 3.4. The vitamin D was extracted from the complex and used in the HPLC procedure. The quantity of vitamin D in the protein vitamin complex was calculated based on the HPLC standard curve. An average concentration of vitamin D in β-LG complex was calculated and used to design a model system for the human intervention study (Chapter 4). Based on the findings from vitamin D content in the powders, sample beverages (targeted vitamin content of 400, 600, 800, and 1000 IU) were manufactured to test if differences (vitamin D content) existed from testing with HPLC versus VitaKit D™. Figure 3.5 shows the standard vitamin D curve created from the internal standards included within the VitaKit D™. Table 3.5, demonstrated that there was not a significant difference between the beverages targeted at 400 IU vitamin D (p=.955) and 800 IU vitamin D (p=.750). However, a significant difference was seen with proposed beverage containing 1000 IU of vitamin D (p=.0076). United States regulations require that vitamin D fortification in acceptable range can be 100 to 150% of the label claims (Murphy et al, 2001). All samples except those made with Star Vitamin and Pat Vitamins were within this regulation. Additionally, binding affinity of vitamin D to β-LG spray-dried in the presence of trehalose and lactose was assessed. The dried powders were extracted according to previous discussion and were analyzed using the VitaKit D™. Data in Table 3.4,
demonstrate that there was no significant difference between the beverages made with β-LG and trehalose versus a similar beverage made with β-LG and lactose.

3.2.4 Discussion and Conclusion

Spray-Drying of β-LG Vitamin Complexes

β-Lactoglobulin has been shown to bind with vitamin D (Kontopidis, 2004; Wang, 1997). This complex has been shown to be an effective fortifier of aqueous solutions (Eledah, 2005 and Reynolds, 2005).

The recovery rate of vitamins was lower than that of protein recovery. The possible causes of low recovery of vitamin may have been due to several factors: 1) The changes in the functional performance of whey protein may be altered due to high temperatures, which may have caused denaturation of protein and affected the binding of the protein to vitamins. 2) The ethanol utilized in experiments may have decreased the percentage of protein-vitamin D binding. 3) Lastly, because vitamin D is usually found in fat-containing systems, the binding of protein to vitamin without an emulsifier in aqueous solution may have been inefficient. Additionally, a 70% protein recovery rate needs to be greatly improved for this particular process to be utilized in industry. A commercial spray drier that is not operating with minimal amounts of protein would likely have a lower percentage of protein loss. Once significant recovery parameters are identified and achieved, spray-drying may be used to prepare β-LG-vitamin complexes more efficiently on a large scale.

The extraction and HPLC methods by Murphy et al. (2001) have been proven as a successful method of determination of vitamin D in fluid milk. Even though these methods were designed for application to a fat-containing substance, we adapted these methods to
quantify vitamin D$_2$ and D$_3$ in an aqueous solution. The HPLC chromatograph has unexpected peaks compared to studies in which a complex matrix (i.e. milk or food component) did not exist (Faulkner, 2000 and Murphy, 2001). These deviations may be due to the use of deionized water as the solvent or noise from the HPLC system. The reproducibility of HPLC chromatographs was erratic. Therefore, additional work should focus on a better analytical method designed to ensure reproducible recovery of vitamin D when utilizing the HPLC procedure. Moreover, due to the fact that vitamin D is fat-soluble, the majority of the vitamin D fortification research is conducted in fat-containing beverages. There are limited published data concerning vitamin D fortification of aqueous solutions or methods for their analysis. This research has shown that the vitamin D and β-LG complex is a good vitamin D carrier to be utilized in aqueous solutions.

Recently, SciMed Technologies (Edmonton, Alberta, Canada) began marketing an ELISA for analysis of vitamin D in milk. There is limited published research that addresses the effectiveness of the SciMed Technologies Inc. VitaKit D™ (ELISA assay) compared to traditional HPLC methodology. HPLC has been seen as the gold standard for assessing vitamin D in various food systems (Ball, 2005). This research indicated there was no significant difference between VitaKit D™ and HPLC measurements of vitamin D content of the aqueous solutions formulated at 400 IU and 800 IU. However, a significant difference between the methods was seen when investigating vitamin D content at 1000 IU, with the ELISA assay producing results closer to the expected value. These findings may have been impacted by use of D$_2$ in the system; the SciMed assay was developed for quantitative determination of vitamin D$_3$ in dairy samples (SciMed Laboratories Inc., Alberta, Canada). The variation may also be due
to analysis at different time points (storage times), which may have contributed to denaturation of vitamin D during storage. The VitaKit D™ procedure was more user-friendly; it reduced the time for vitamin D evaluation in food systems and increased the number of samples that could be assayed at one time. Further research should investigate if an ELISA assay method is appropriate for assessing vitamin D content in other food systems and improve accuracy and accountability for vitamin D testing.

The improvement of functional properties of proteins using various sugars, lactose and trehalose was investigated. Trehalose and lactose are both disaccharides that have been shown to have a protective effect in spray drying procedures (Liao et al., 2002). No difference was observed between use of lactose compared to trehalose for vitamin D protection during spray drying, which differs from results from previous studies (Liao et al., 2002 and Richards et al., 2002) showing that trehalose had a greater preservation effect on proteins than lactose. While results from this study differed from previous studies, the outcomes from this study might have been due to low recovery of vitamin verses protein. The sugars may have been unable to protect the conformational structure of the protein which in turn did not allow for binding of the vitamin to the protein structure. However, this research did illustrate the potentially complex nature of the impact of sugars (lactose and trehalose) on the structural preservation of vitamin D in spray drying (Semenova et al., 2002). There is a need for further research on the effects of sugars on preservation of protein and bound vitamins during spray drying and effect of the various sugars on absorption of ligands in animal and human studies (Richards et al., 2002).
These findings helped facilitate the development of a model vitamin D fortifier for use in an aqueous solution. The fortifier complex was tested for bioavailability in a human intervention study (Chapter 4).

3.3 Protein-Vitamin Binding – Fluorescence Study

3.3.1 Introduction

Beta-lactoglobulin (β-LG) has many important functions; one of the main functions is its ability to bind fat-soluble compounds including vitamin A palmitate and vitamin D₃ (Wang, 1999). Research has shown that vitamin D is bound within the β-barrel of the molecule. The topography of β-lactoglobulin allows for physical shelter of hydrophobic molecules from oxidation and heat (Yang et al., 2009). Moreover, it has been suggested that β-LG is the potential carrier of vitamins in reduced-fat milk. The β-LG molecule structurally has two hydrophobic pockets that are capable of binding lipophilic molecules. Crystallography and fluorescence spectroscopy suggest that the sites of binding are the calyx formed by the β-barrel and the other between the α-helix and the surface of the barrel, as illustrated in Figure 3.3 (Yang et al., 2009). The purpose of this study was to validate the binding of β-LG to vitamin D utilizing fluorescence technology. Previously, Dufour et al. (1990) illustrated fluorescence emission of Tryptophanyl 19 (Trp 19) at the bottom of the calyx is quenched at 332 nm when a ligand is bound; this information was employed to characterize the binding of β-LG to vitamin D.
Figure 3.3 Vitamin D Transport and Binding by Beta-lactoglobulin

Amino acid sequence of LG and crystal structure of the LG–vitamin D₃ complex. (A) LG comprises 162 amino acids with nine β-sheet strands (A–I). The α-helix with three turns is located between residues 130 and 141 (yellow). (B) Space-filling drawing of the LG–vitamin D₃ complex at 2.4 Å resolution. Vitamin D₃ (carbon yellow and oxygen red) and LG are drawn on the basis of our previously refined model with one vitamin D₃ molecule penetrating inside the calyx (left) and the other lying on the surface pocket at the C-terminus (residues 136–149) (right). (C) Front view of vitamin D₃ binding to the exosite and chemical structure of vitamin D₃. (D) The 3D ribbon model of the LG–vitamin D₃ complex shows that the exosite combines an α-helix (red) and β-strand I (pink) with a α-turn loop (green). (Yong et al., 2009)
3.3.2 Materials and Methods

Bio-pure β-LG (95% of total protein) was procured from Davisco Food International, Inc. (Le Sueur, MN). Vitamin D₃ powder (MW 384.65) and vitamin D₂ powder (MW 396.65) were purchased from Sigma Chemical Company (St. Louis, MO) and Roche Vitamins Inc. (Nutley, NJ), respectively. Additionally, vitamin D₃ liquid and vitamin D₂ liquid were supplied by Freeman Industries, LLC (Tuckahoe, NY). The other high quality chemicals used in the analysis were purchased from Sigma (St. Louis, Mo) or Fisher (Pittsburgh, PA). The instrumentation was completed on a System 3 Scanning Spectrofluorometer (Optical Technology Devices, Inc., Elmsford, NY) to verify the binding of the protein with vitamin D. Fluorescence emissions were measured with 1 µm solutions of β-LG in 50 mM sodium phosphate, at pH 7.0, both in the presence and absences of various ligands. As stated earlier, the binding ability was determined by measuring the fluorescence quenching of the tryptophan residue T19. To test each ligand, 2.5 mL of 1 µM β-LG in phosphate buffer was contained in a spectrofluorometer cuvette to which 2.5 µL increments of ligand solution in ethanol were added. This procedure was performed at 21°C using an excitation wavelength of 287 nm and a scan speed of 50 nm/min. A solution of N-actyl-tryptophanamide with an absorbance at 287nm; which was equivalent to the protein solution was titrated in a similar manner to serve as the control (blank) sample. The increased fluorescence intensity quenching at 332nm is quantitatively related to vitamin D binding (Wang et al., 1997).
3.3.3 Results

Protein-Vitamin Binding –Fluorescence Spectroscopy Study

Fluorescence Spectroscopy was used to verify the binding of vitamin D to protein. Fluorescence emission was conducted in the presence and absence of vitamin D from various sources. The procedure was performed at 21°C using an excitation wavelength of 280 nm and a scan speed of 50 nm/min. The intensity of fluorescence increased at approximately 284 nm and fluorescence quenching at 332 nm for all samples (Figures 3.6-3.7). These findings are quantitatively related to results that have been seen previously with retinyl palmitate binding (Wang et al., 1999).

3.3.4 Discussion and Conclusion

Protein-Vitamin Binding –Fluorescence Spectroscopy Study

The binding of vitamin D with BioPure β-LG was confirmed by quenching of the protein fluorescence at wavelength 332 nm. These data, fluorescence emission spectra, were similar to those of β-LG binding with ligand as seen in previous research (Wang, 1999) and are consistent with the model of binding in the β-barrel near Trp-19. The characterization of the binding of protein with ligand suggested that the BioPure β-LG and the emulsified vitamin D form complexes for use as fortifiers. The characterization, at a molecular level, of the β-LG binding sites(s) by fluorescence spectroscopy indicated that β-LG is capable of binding at least 1 mole of vitamin A or D per mole when pure protein and vitamins were used in preparation of protein-vitamin complexes (Dufour, 1990; Wang, 1999).
3.4  Shelf-Life and Milk Comparison Study

3.4.1  Introduction

Since the 1930s vitamin D added to fluid milk has been utilized to reduce the prevalence of rickets in the United States. Currently, milk fortification is regulated by the Pasteurized Milk Ordinance, which requires the mandatory fortification with vitamin A in non-fat and reduced fat milk. This ordinance does not address fortification with vitamin D; vitamin D fortification is not mandatory like vitamin A. However, when added, milk should specify a content of 400 (I.U.) per quart, and acceptable levels of vitamin D fortification range from 400 to 600 I.U. per quart. Multiple studies have concluded that a high percentage of fortified milk products are out of compliance, usually with under-fortification with vitamin D (Faulkner et al., 2000; Holick 1992; Murphy et al., 2001; and Nichols, 1992). Under-fortification of vitamins in milk has been attributed to loss of vitamin during processing and storage. Vitamins are sensitive to light, high temperatures (heat), and oxygen. Researchers have suggested that fat content plays a significant role in the preservation of vitamins. It has been suggested that milk fat appears to protect against vitamin degradation, therefore as fat content decreases it tends to lead to under-fortified milk on the market (Faulkner et al., 2000). Beta-lactoglobulin is proposed to be a stable and protective fortifier in low fat containing beverages based on its ability to bind to vitamin A and D. The purpose of this experiment was to address stability of β-LG -complexed vitamin within a 7-day period (the greatest amount of time beverages would be held in the human intervention study before consumption). A second objective of this experiment was to compare the amount of vitamin D in the β-LG -complex vitamin D solution with various fat-content milks on the market.
3.4.2 Materials and Methods

Vitamin D fortified milk (whole, 2% reduced fat, 1% low fat and skim milk) was purchased from a local grocery store in Raleigh, NC. The vitamin D content was tested by the VitaKit D™, procedure as discussed above. Commercial milk samples were packaged in high-density polyethylene (HDPE), except for whole milk sample, which was contained in a paperboard carton. The whole milk had a sell by date of November 1, 2009, 2% reduced fat milk of October 25, 2009, 1% lowfat milk October 24, 2009 and skim milk sell by date of October 25, 2009. Both β-LG vitamin D spray-dried complex and β-LG-vitamin D complex non spray-dried were used for beverage formulation in our lab. The other food grade reagents used in the beverage solution were purchased from a Sigma Chemical Co. (St. Louis, MO), Fisher (Pittsburgh, PA), and a local grocery store (Raleigh, NC). The formulation of the beverage included β-LG vitamin D complex, raspberry flavor, sodium benzoate, non-caloric sweetener, and deionized water. The production of beverages occurred in a food grade facility at North Carolina State University (Raleigh, NC). The ingredients were combined and cold filled in pasteurized, lined metal cans (House of Cans, Lincolnwood, IL). The cans were sealed with Dixie Automatic Can Sealer (Dixie Canner Company, Athens, GA) and stored at refrigeration temperature (4°C) (Low Temperature Incubator 815, Thermo Electron Corporation, Waltham, MA) for 7 days. During the shelf life study aerobic bacteria counts were taken utilizing 3M™ Petrifilm™- Aerobic Plates (St. Paul, MN). One mL of each beverage solution was inoculated on duplicate 3M™ Petrifilm. The samples were incubated at 7°C for 2 to 7 days. The plates were enumerated by counting the red colonies at time point 1 day, 2 days, and 7 days after beverage
preparation. The use of coated metal cans and microbiological testing were critical control points to ensure the safety of the products for human consumption.

3.4.3 Results

Shelf-Life and Milk Comparison Study

Shelf-life results for different β-LG vitamin D complexes are presented in Table 3.6. The beverages were fortified with a 30% increase over calculated value to ensure enough vitamin D would be present after formulation and storage. Table 3.6, illustrates the amount of vitamin D remaining in the beverage after 7 days of storage at 4°C. The findings listed illustrated that some formulations were able to maintain vitamin D status after storage. This information was utilized to help manufacture beverages of the human intervention study.

The results for the aerobic bacteria growth are shown in Figures 3.8-3.9. As illustrated, the aerobic bacteria were above safe levels for human consumption. To assess the source of contaminants, all food grade ingredients utilized in the beverage and stock solution were also tested. This analysis revealed no growth of bacteria, as illustrated by Figure 3.9 in the stock solution. A comparative study was completed for spray-dried β-LG vitamin D complex against beverages that contained all ingredients but was not spray-dried. Once again aerobic bacteria growth was significantly higher in beverages that utilized spray-dried material compared to non-spray-dried. Food safe for human consumption may contain 25-250 cfu/mL (Codex, Alimetarius, 1997). Therefore spray-dried samples were not utilized in the human intervention study. Moreover, vitamin D content was tested on non spray-dried beverages and fluorescence spectroscopy showed that vitamin D was able to bind to the protein via fluorescence spectroscopy.
The results for the vitamin D analyses in milk and fortified beverage are shown in Table 3.7. For vitamin D, a total of 4 milk samples and 2 research beverage samples were selected for analyses, a total of 24 samples (4 samples per beverage) were tested for vitamin D. All of the milks tested were fortified with both vitamin A and D. Table 3.7 is a summary of the results of all fortified milks tested. Based upon regulations 2 out of the 4 milk samples were within the acceptable ranges for vitamin D (400-600IU). On the other hand, if the research beverage were assessed utilizing these same standards then none of these samples would be within the acceptable range. Most samples tested were out of compliance with regulatory guideline. Whole milk and 2% reduced fat milk were within compliance, but beverages with lower fat content (1%, skim milk, and aqueous) had vitamin D content lower than the targeted level. The aqueous beverage fortified with β-LG-vitamin D complex had significantly more vitamin D than skim milk, and an amount that was not different from 1% fat milk. However, this information was used to make research beverages that were within the regulatory guidelines and provided the amount of vitamin D needed for a human intervention study.

3.4.4 Discussion and Conclusion

Shelf-Life and Milk Comparison Study

For the duration (7-day period) of the shelf-life study the results illustrated no appreciable changes or differences between vitamin D$_2$ and vitamin D$_3$, indicating that both vitamers are acceptable for fortification of milk. These findings differ from studies that have stated vitamin D$_3$ is more effective than D$_2$ (Houghton and Vieth, 2006 and Armas et al., 2004). Moreover, the study illustrated that achieving targeted vitamin D levels in an aqueous solution and maintaining vitamin D content was possible. Vitamin D concentration decreased and had
values below targeted parameters in samples fortified with vitamin D from sample VD53, a vitamin D powder, and VD29, a vitamin D3 400,000 IU/G powder, respectfully. This may have been due to unknown fillers or other additives. This experiment did observe samples that were not in compliance with targeted vitamin D parameters, this has also been seen with milk research studies that have evaluated vitamin D content in commercial milks (Murphy et al., 2001).

The vitamin D levels were assessed in both the commercially available milk products and the research vitamin D fortified beverages. The vitamin D content in the whole and 2% reduced fat milk were within regulation. However, the remaining milk samples and research beverages were below regulated levels, which have also been seen in other studies that have investigated vitamin D content in fluid milk (Murphy et al., 2001, Faulkner et al., 2000, and Hicks et al., 1996). Most of the samples out of compliance with vitamin D were below the acceptable limits. The 1% lowfat milk samples and both research beverage samples were within 20% of the label claim. However, the skim milk samples were approximately 40% below label claim. These errors or underfortification may be attributed to error in method of fortification, possible vitamin degradation, oxidation and potential variability in results of vitamin analyses (Murphy et al., 2001).

For future work, to completely compare the research beverages to commercial milk samples, the research beverages should be contained in similar medium as seen in commercial milk samples such as paperboard cartons or polyethylene. This would test the ability of β-LG to protect vitamin deterioration under similar conditions as the milk beverages. Research investigating proper vitamin D fortification among milk beverages must continue to ensure
compliance. Adequate vitamin fortification in milk may play a significant role in preventing vitamin D deficiency and insufficiency in the United States (Murphy et al., 2001).
Figure 3.4 Vitamin D Standard Curve

y = 0.1149x - 0.0825
$R^2 = 0.9922$
Figure 3.5 Vitamin D Standard Curve Vitakit D™
Table 3.4 Protein-Vitamin Binding – Trehalose verses Lactose Comparison

<table>
<thead>
<tr>
<th></th>
<th>Vitamin D/ Lactose (spray dried)</th>
<th>Vitamin D/ Trehalose (spray dried)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>631.22</td>
<td>671.33</td>
</tr>
<tr>
<td>Observations</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>t Stat</td>
<td>-0.127754478</td>
<td></td>
</tr>
<tr>
<td>P(T&lt;=t) one-tail</td>
<td>0.451258781</td>
<td></td>
</tr>
<tr>
<td>t Critical one-tail</td>
<td>1.943180274</td>
<td></td>
</tr>
<tr>
<td>P(T&lt;=t) two-tail</td>
<td>0.902517562</td>
<td></td>
</tr>
<tr>
<td>t Critical two-tail</td>
<td>2.446911846</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.5 Comparative Analyses High Performance Liquid Chromatography (HPLC) verses Vitakit D™

<table>
<thead>
<tr>
<th>Proposed Vitamin D (IU)</th>
<th>HPLC Actual Vitamin D (IU) (Mean ± SD)</th>
<th>VitaKit D™ Actual Vitamin D (IU) (Mean ± SD)</th>
<th>N</th>
<th>F</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>408.00±299.76</td>
<td>410.93±26.88</td>
<td>8</td>
<td>.0034</td>
<td>.955</td>
</tr>
<tr>
<td>800</td>
<td>812.00±899.27</td>
<td>793.44 ±7.76</td>
<td>8</td>
<td>.0111</td>
<td>.750</td>
</tr>
<tr>
<td>1000</td>
<td>1608.08±859.32</td>
<td>1025.43±115.52</td>
<td>8</td>
<td>15.46</td>
<td>p&lt;.05**</td>
</tr>
</tbody>
</table>

Figure 3.6 Fluorescence emission spectrum of BLG in the absence (a) and presence (b) of D$_2$ powder, (c) D$_3$ powder, (d) D$_2$ liquid, and (e) D$_3$ liquid.
Figure 3.7 Fluorescence emission spectrum of BLG in the absence (a) and presence (b) of D$_2$ powder (c) D$_3$ powder, (top panel); and fluorescence emission spectrum of BLG in the absence (a) and presence (d) D$_2$ liquid, and (e) D$_3$ liquid (bottom panel)
Table 3.6 Shelf–Life: Vitamin D Content after 7-Day Storage

<table>
<thead>
<tr>
<th>Sample (IU/250ml)</th>
<th>200 IU</th>
<th>400 IU</th>
<th>600 IU</th>
<th>1000 IU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Vitamin D</td>
<td>260.43</td>
<td>520.10</td>
<td>780.00</td>
<td>1300.79</td>
</tr>
<tr>
<td>Sigma Vitamin D$_3$/ Lactose (spray dried)</td>
<td>238.72</td>
<td>467.42</td>
<td>625.15</td>
<td>1193.58</td>
</tr>
<tr>
<td>Sigma Vitamin D$_3$/ Trehalose (spray dried)</td>
<td>271.39</td>
<td>442.78</td>
<td>614.17</td>
<td>1256.96</td>
</tr>
<tr>
<td>Roche Vitamin D$_2$/ Lactose</td>
<td>290.86</td>
<td>413.71</td>
<td>627.57</td>
<td>1354.28</td>
</tr>
<tr>
<td>Pat Vitamins D$_3$(VD/53)/Lactose</td>
<td>159.91</td>
<td>319.82</td>
<td>479.72</td>
<td>799.54</td>
</tr>
<tr>
<td>Danisco D$_3$(VD/29)/Lactose</td>
<td>189.77</td>
<td>379.54</td>
<td>569.31</td>
<td>948.85</td>
</tr>
</tbody>
</table>
Table 3.7 Milk Comparisons – Vitamin D Content of Commercially Available Milk versus Research Beverages

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (IU/mL)</th>
<th>Concentration (IU/cup)</th>
<th>Concentration±S.D. (IU/4cups)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole milk</td>
<td>0.502</td>
<td>118.397</td>
<td>473.59±99.78^a</td>
</tr>
<tr>
<td>2% Reduced Fat Milk</td>
<td>0.452</td>
<td>106.566</td>
<td>426.26±160.39^b</td>
</tr>
<tr>
<td>1% Lowfat Milk</td>
<td>0.396</td>
<td>93.567</td>
<td>374.27±36.51^c</td>
</tr>
<tr>
<td>Skim Milk</td>
<td>0.295</td>
<td>69.560</td>
<td>278.24±148.76^d</td>
</tr>
<tr>
<td>Research Beverage D$_2$</td>
<td>0.374</td>
<td>88.170</td>
<td>352.68±178.31^c</td>
</tr>
<tr>
<td>Research Beverage D$_3$</td>
<td>0.402</td>
<td>94.852</td>
<td>379.41±205.03^e</td>
</tr>
</tbody>
</table>
Figure 3.8 Aerobic Bacteria – β-LG Vitamin D Complex
Figure 3.9 Aerobic Bacteria – Stock Solution and Non-Spray Dried Beverage Complete
Figure 3.10 Aerobic Bacteria Spray Dried (middle and bottom) verse Non Spray Dried (top) (Initial (middle) and 7-day Storage (bottom)}
REFERENCES


CHAPTER 4

Fortification of an Aqueous Solution with β-LG Vitamin D Complex: Human Bioavailability

Mallorye D. Lovett

Department of Food, Bioprocessing and Nutrition Sciences

North Carolina State University
ABSTRACT

Growing awareness of vitamin D status in the United States has resurfaced due to increasing reports of insufficiency and deficiency. Epidemiological research indicates that average intake of this nutrient is well below the RDA, and greater intake has been linked to optimal bone health, prevention of osteoporosis, osteomalacia, and rickets and some other chronic diseases.

The objective of this study was to test a water-soluble form of vitamin D as a fortifier for an aqueous beverage with a human bioavailability assay.

A water-soluble vitamin D fortifying ingredient was prepared as a complex with bovine beta-lactoglobulin. Vitamin D content of the complex was assessed by HPLC and VitaKit D™. Flavored beverages were formulated with 400 IU, 600 IU, and 1000 IU of vitamin D. Human subjects recruited from North Carolina State University (Raleigh, NC) participated in a blind randomized clinical trial designed to determine the bioavailability of vitamin D₂ versus D₃ at various levels. Subjects consumed 8 fl. oz of a vitamin D beta-lactoglobulin complex-fortified beverage at each trial (total 5 trials) and blood was drawn at baseline, 4, 8, 24 hours post-consumption. Serum vitamin D was measured by ELISA.

Results show that aqueous sports drink solutions could be accurately formulated to contain vitamin D (400 IU, 600 IU, and 1000 IU) with palatability to humans. Serum vitamin D analysis demonstrated a significant difference (p<0.0001) in serum vitamin D levels by race. Moreover, there was an increase in blood serum levels for all participants from initial treatment to final treatment. Subjects did not respond differently to drinks containing vitamin D₂ or D₃.
Water-soluble vitamin-D can be used to fortify aqueous products and consumption can help facilitate the uptake of vitamin into the blood. Regular consumption of flavored drink fortified with vitamin D can significantly increase dietary vitamin D intake.
4.1 Fortification of an Aqueous Solution with β-LG Vitamin D Complex: Human Study

4.1.1 Introduction

Vitamin D deficiency and insufficiency remains a public health issue. Whereas vitamin D insufficiency is largely unrecognized, it has the ability to increase the prevalence of bone abnormalities and development of certain chronic diseases (Wagner et al., 2008). Vitamin D plays a critical role in the homeostatic control of calcium and phosphorus. Thus, adequate intake of vitamin D increases the efficiency of intestinal calcium absorption, which facilitates skeletal development (Holick, 2005). The purpose of this study was to examine the effects of β-LG vitamin D complex as fortifiers for an aqueous sports drink solution with a human bioavailability intervention study. Vitamin D plays a critical role in the prevention of metabolic diseases including osteoporosis, osteomalacia, rickets, and other chronic diseases, including certain cancers, diabetes, and cardiovascular disease (Wagner et al., 2008). Epidemiological research indicates that average intake of this nutrient is well below the RDA, and greater intake has been correlated to a reduction in fractures, prevention of osteoporosis, and increased bone mass (Bischoff-Ferrari, 2006).

Vitamin D can be obtained by action of sunlight on the skin and the diet. Exposure to the UV rays from the sun induces a photolytic conversion of 7-dehydrocholesterol to previtamin D₃ which is further hydroxylated to the active usable form of vitamin D. Furthermore, the diet only has a few natural food sources that contain significant amounts of vitamin D₂ and D₃. Therefore, in the 1930s vitamin D fortification was employed to alleviate and address the issue of vitamin D deficiency. Several food items during this period were fortified with vitamin D
including milk, breads, hot dogs, sodas, and even alcoholic beverages including beer (Tangpricha et al., 2003). With the abundance of various vitamin D fortified foods, intoxication of vitamin D was identified in certain Europeans countries. The United States Food and Drug Administration proactively regulated fortification in the US, limiting vitamin D fortification to cereals and milk (Wagner et al., 2008; Tangpricha et al., 2003). This regulation in the 1950s almost totally eradicated vitamin D deficiency and rickets in the United States (Wagner et al., 2008). Today in the 21st century vitamin D deficiency has reemerged (Nield et al., 2006).

Insufficient intake of vitamin D has led to the re-emergence of vitamin D dependent rickets in some US populations, especially darkly pigmented people, breastfed infants, and individuals living at higher latitudes (Nield et al., 2006). Due to the re-emergence of rickets and the deficient intake of vitamin D, experts have stated that current recommendations for vitamin D intake are too low. Therefore, these experts are recommending increasing the RDA values for vitamin D (Hathcock, 2007). Current randomized supplementary trials utilizing the current RDA for vitamin D, 200 IU, have not shown sufficient reduction in fracture risk (Vieth, 2007). On the other hand, other research studies have shown supplementation above the current RDA yielded a decrease in fractures (Holick, 1998, Hollis, 2004 & Vieth, 2007). These research studies also lay the framework for more fortification of various food products with vitamin D.

There is strong evidence indicating that current levels of fortification are inadequate to address vitamin D deficiency and insufficiency (Harris et al., 2000). In addition, one of the main sources of vitamin D through fortification, milk, frequently contains less than the stated amount (Faulkner et al., 2000, Holick 1992, Murphy et al., 2001, and Nichols, 1992). Additionally, milk consumption has also been declining (Holick, 1992). These findings have fueled the need for
fortification of other food sources with vitamin D. Recently, bottled water consumption has seen exponential growth (Decker, 2006), which suggests that a vitamin D-fortified bottled water could increase vitamin D intake by many people. Because vitamin D is a fat-soluble vitamin, it is easiest to add vitamin D to fat-containing systems. The current study investigates whether β-LG vitamin D complex has an effect on the bioavailability in healthy adults. Changes in serum concentrations of 25-hydroxyvitamin D [25(OH)D] were measured as a biomarker for vitamin D bioavailability in volunteers consuming an aqueous solution fortified with β-LG-vitamin D complex.

4.2 Materials and Methods

USP-grade cholecalciferol D3'Sol and ergocalciferol D'Sol were procured from Freeman Industries LLC (Tuckhoe, NY); quantitation of vitamin D was ascertained (Chapter 3). The calculated vitamin D content (D’3 Sol or D’Sol) was added to a formulation which included deionized water, beta-lactoglobulin, lactose, fruit flavor, sodium benzoate, and non caloric sweetener. The beverages were made in a food grade facility at North Carolina State University (Raleigh, NC). The ingredients were combined and cold filled in pasteurized lined metal cans (House of Cans, Lincolnwood, IL). The cans were sealed with Dixie Automatic Can Sealer (Dixie Canner Company, Athens, GA) and stored at refrigeration temperature (4°C) (Low Temperature Incubator 815, Thermo Electron Corporation, Waltham, MA) for no more than 7 days. Analysis was conducted to ensure appropriate vitamin D content by VitaKit D™, as described in Chapter 3.
Recruitment

The Institutional Review Board at North Carolina State University (Raleigh, North Carolina) approved the research study. All study subjects gave written informed consent for participation in this research.

Subjects were recruited through the use of an internet e-mail at North Carolina State University (Raleigh, North Carolina) (Appendix A). Healthy men and women between the 18 and 50 years of age were candidates for inclusion in the study. They were instructed to complete an anonymous computerized questionnaire to determine eligibility for participation (Appendix B). They were not admitted to the study if any of the following criteria were present: 1) a history of any medical disorders that might affect vitamin D metabolism; 2) use of medications that could interfere with vitamin D metabolism; 3) use of calcium supplements or antacids containing calcium; 4) pregnant women; 5) taking medications that require consumption of food in the morning; 6) individuals with a lactose allergy or lactose intolerance; 7) potential for significant sun exposure (i.e. travel to a sunny destinations or use of tanning beds; or 8) unwillingness to repeat the experiment as the protocol indicated. Between March and April 2009, 43 individuals met the eligibility requirements, most of whom were students at North Carolina State University (Raleigh, NC) and associated with the Department of Food, Bioprocessing, and Nutrition Sciences (North Carolina State University, Raleigh, NC). Each individual was contacted by a follow-up e-mail that gave additional information and a copy of the consent form that had to be signed in front of the investigator on the first day of the study (Appendix C). Upon arrival participants received a modified food frequency questionnaire to assess vitamin D. This modified food frequency questionnaire has been shown an effective tool to monitor calcium and
vitamin D status (Blalock et al., 2003). Measuring cups were provided as a visual aid to determine serving size. The questionnaire was completed at the beginning of study and at the completion of study.

**Study Design**

The 43 (16 male, 27 female) eligible subjects were randomly assigned beverage order prior to coming to the intervention. Randomization sequence was generated by the investigator to produce randomly permutated blocks. Study 1: A blind randomized clinical trial designed to determine the bioavailability of vitamin D$_2$ versus D$_3$. Subjects consumed 8 fl oz of the following treatments: control (unfortified drink), intervention 1 (control + vitamin D$_2$ (400 IU)), and intervention 2 (control + vitamin D$_3$ (400 IU)). Fasting blood samples were collected from all study participants at the time of enrollment. A 500-µL serum sample was drawn by each individual by finger prick (finger prick procedure instructions were given to subjects). In a preliminary trial in which venous blood samples were compared to finger prick blood samples, no significant difference was seen. Each finger prick should yield approximately 0.5 mL of blood, a total of 2 mL for each treatment at various time intervals, and a total of 10 mL for the entire study. Blood samples were collected at 0, 6, 12, and 24 hours post-consumption. The samples were separated, aliquoted, and frozen at -70ºC until samples were analyzed for serum 25-OH vitamin D. Transient rises in serum 25-OH vitamin D during the 24 hr following the oral dose were predicted based on the study by Johnson et al., (2005).

Study 2: Based upon the results of the first feeding trial, one beverage combination was selected for the subsequent trial. This study investigated the effects of increasing amounts of vitamin D at 600 IU, and 1000 IU on serum 25-OH vitamin D. Fasting blood samples were
collected from all study participants at the start of the study. Blood samples were collected 6, 12, and 24 hr post-consumption. A 500-µL blood sample was drawn by finger prick, separated, aliquoted, and frozen at -70°C for the duration of the study, at which point the samples were analyzed for serum 25-OH vitamin D.

After completion of the first week of the study, modifications were made to increase retention of study participants. Only 11 out of the 43 eligible individuals completed the first trial. Therefore, blood collection times were changed to 0, 4, 8, and 24 hours and recruitment email was redistributed to increase participation. In addition, the feeding trials were altered to a blind randomized clinical trial designed to determine the bioavailability of vitamin D₂ versus D₃. The second week of the intervention trial subjects received either 600 IU of D₂ or D₃. During the remainder of the intervention trial period, subjects consumed 8 fl oz of the following treatments: control (unfortified drink), intervention 1 (control + vitamin D₂ (1000 IU)), and intervention 2 (control + vitamin D₃ (1000 IU)). Fasting blood samples were collected from all study participants at the time of enrollment. A 500-µL serum sample was drawn by each individual by finger prick (finger prick instructions were given to subjects). Each finger prick should yield approximately 0.5 mL of blood, a total of 2 mL for each treatment at various time intervals, and a total of 10 mL for the entire study. Blood samples were collected at 0, 4, 8, and 24 hours post-consumption. The samples were separated, aliquoted, and frozen at -70°C until samples were analyzed for serum 25-OH vitamin D.

The protocol began in the fourth week of April and ended the fourth week of July. Subjects came to North Carolina State University (Raleigh, NC) for a total of 5 trials (20 visits). At each visit, subjects supplied blood samples for biochemical testing and each subject was
provided with the \( \beta\)-LG vitamin D fortified research beverage to consume within 30 minutes after opening.

Previous research studies have determined that serum 25(OH) vitamin D metabolite is the appropriate assessment of vitamin D status (Holick, 1999). Thus, 25(OH) vitamin D metabolite measurement was utilized to assess bioavailability from the aqueous vehicle and various sources of vitamin D (D\(_2\) and D\(_3\)). The primary outcome was the comparison between sources of vitamin D, treatment groups, and differences in serum levels after consumption of the fortified beverage. The following hypotheses, which were set before the beginning of the study, were tested: 1) the change in serum 25(OH) vitamin D (from baseline to completion of the study) will be significantly greater; 2) the change in serum 25(OH) vitamin D will not differ among vitamin D sources; and 3) the change in serum 25(OH) vitamin D will be significantly greater in the vitamin D-treated samples compared with control.

4.2.1 Analysis for Serum 25-OH Vitamin D\(_3\)

An Octeia 25-Hydroxy Vitamin D Elisa Kit (IDS Inc., Fountain Hills, AZ) was used to analyze serum 25-OH vitamin D. This kit is approved by the Food and Drug Administration (FDA) for analyzing 25(OH) vitamin D status.

The labeled serum samples were removed from -20°C storage and placed in a room temperature water bath for 2-3 minutes. The protocol followed was in accordance with the manufacturers’ product insert. Polypropylene test tubes were labeled for each calibrator, control and sample. Twenty-five \( \mu\)L of each calibrator, control and sample were added to the appropriate test tube. One mL of 25-D biotin solution was added to all the test tubes. Each test tube was vortexed for approximately 10 seconds. Two hundred \( \mu\)L of each diluted calibrator,
control and sample were added to the appropriate well of the antibody-coated plate in duplicate. After the samples were added to the plate, the lid was placed on the plate and sealed in a plastic bag. The plate was incubated at 18-25°C for 2 hours. Following, the incubation period the plate wells were washed manually with 250 µL of buffer solution provided. The buffer solution was decanted and the procedure repeated twice. The plate was inverted firmly on absorbent tissue to remove excess fluid. Two hundred µL of enzyme conjugate was added to each well. The lid was placed upon the plate again and incubated at 18-25°C for 30 minutes. The manual wash step was repeated. After the plate was firmly inverted to remove excess buffer solution, 200 µL of tetramethylbenzidine substrate was added to each well using a multichannel pipette. The plate was again sealed and incubated at 18-25°C for 30 minutes. One hundred µL of 0.5 M HCl was added to each well with a multichannel pipette to stop the reaction. Within 30 minutes of adding the 0.5 M HCl the absorbance was measured at 450 nm using a microplate reader (Thermo Electron Corporation, Vantaa, Finland).

4.2.2 Statistical Analysis

SAS software (Cary, NC) was used for the statistical analysis of the data. Moreover, this software was employed to determine the correlation between vitamin D in water and serum vitamin D levels. Comparative analysis was conducted between various treatment groups. The serum vitamin D concentrations in the β-LG vitamin D beverages were analyzed by repeated measures analysis of variance (ANOVA). The serum 25(OH) vitamin D concentrations in the research beverages were analyzed by ANOVA in both the β-LG-vitamin D beverages and the control beverages. Further analyses were performed with Bonferroni techniques to determine
difference in serum 25 (OH) vitamin D concentrations at several time points compared with baseline values

4.3 Results

Eighteen subjects enrolled in the study, of whom 14 (77%) completed the entire protocol with 0 missed visits. After time and trial modifications, 16 (88%) study participants completed the remainder of the study. The ages of the participants ranged from 18-50 years of age [18-24 yr (5 subjects), 25-29 yr (6 subjects), 30-39 yr (6 subjects), and 40-50 yr (1 subject)]. Reasons for nonparticipation included being away from the university on the day of measurements, changes in work schedules, and an unwillingness to participate due to finger prick blood draws. Approximately, 55.6% (10 subjects) were females with the remaining 44.4% (8) males. Of them, 70% of the participants were Caucasian, 24.2% African American and 5.8% Asian. For statistical analysis the groups, African American and Asian were combined into the one group entitled minorities (30.0%).

Dietary intake of calcium and vitamin D were determined by a modified food frequency questionnaire, which has previously been shown to provide reliable results (Blalock et al., 2003). Participants were instructed that no dietary modifications were necessary for the duration of the study. The participants were instructed by a Registered Dietitian (RD) at the beginning of the study to reflect upon their eating habits and record their dietary intake. Measuring cups and measuring spoons were supplied to facilitate proper measurement estimation. In addition, at the end of the study participants were again asked to complete the modified food frequency questionnaire and reflect upon the last months they were enrolled in the study. The records
were reviewed by the RD and calcium and vitamin D quantities were determined by the United States Department of Agriculture (USDA) Nutrient Data Laboratory computerized system. This system translates a given quantity of a certain food into a composition profile including information such as vitamin and mineral content. As illustrated in Tables 4.1-4.2, a medium serving size was used to assess micronutrient contents of selected food items. The information gathered was used to extrapolate daily dietary intake of both calcium and vitamin D of each participant, as shown in Table 4.3. In addition, the calcium and vitamin D intake was calculated at the beginning and end of the study with the self-reported vitamin D content in a multi-vitamin. The average calculated dietary intake of calcium and vitamin D were below the recommended daily intakes, 1000 mg calcium and 200 IU vitamin D, at the beginning and end of the study. Excluding the amount of vitamin D contributed by a multi-vitamin, only 16.6% of individuals had adequate daily intake of calcium and vitamin D at the start, whereas 11.1% had adequate intake of calcium and vitamin D at the end of the study. Including the amount of vitamin D contributed by a multivitamin, 27.7% of the participants had adequate intake of vitamin D. Individuals using a multi-vitamin reported consuming the multi-vitamin for several months and for the duration of the study.

There was no significant difference in calculated dietary intake among gender or racial categories. More than half of the subjects consumed less than one glass (8 fl. oz) milk per day. Reported milk consumption did differ significantly by race; minorities consumed a lower amount of milk compared to their Caucasian counterparts. Regular consumption of multivitamin containing vitamin D was reported by three participants, all women.
Table 4.1 Modified Food Frequency Questionnaire for Assessing Dietary Intakes of Calcium and Vitamin D in Beverages

<table>
<thead>
<tr>
<th>Type of Beverage</th>
<th>Medium Serving</th>
<th>Calcium mg</th>
<th>Vitamin D IU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole milk and beverages with whole milk (not including on cereal)</td>
<td>8 fl oz glass</td>
<td>349</td>
<td>98</td>
</tr>
<tr>
<td>2% milk and beverages with 2% milk (not including on cereal)</td>
<td>8 fl oz glass</td>
<td>349</td>
<td>98</td>
</tr>
<tr>
<td>Skim milk, 1% milk, or buttermilk (not including on cereal)</td>
<td>8 fl oz glass</td>
<td>349</td>
<td>98</td>
</tr>
<tr>
<td>Milk in coffee or tea</td>
<td>1 tablespoon</td>
<td>21</td>
<td>6</td>
</tr>
</tbody>
</table>
Table 4.2 Modified Food Frequency Questionnaire for Assessing Dietary Intakes of Calcium and Vitamin D in Food Items

<table>
<thead>
<tr>
<th>Type of Food</th>
<th>Medium Serving</th>
<th>Nutrient Amount</th>
<th>Calcium mg</th>
<th>Vitamin D IU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broccoli</td>
<td>1/2 cup</td>
<td>104</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Mustard greens, turnip greens, collard greens</td>
<td>1 cup</td>
<td>102</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Spaghetti, lasagna, other pasta with tomato sauce</td>
<td>1 cup</td>
<td>102</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Mixed dishes with cheese (such as macaroni and cheese)</td>
<td>1 cup</td>
<td>264</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td>White bread (including sandwiches, bagels, burger rolls, French or Italian bread)</td>
<td>2 slices</td>
<td>76</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Dark bread, such as wheat, rye, pumpernickel (including sandwiches)</td>
<td>2 slices</td>
<td>71</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Biscuits or muffins (including fast foods)</td>
<td>1 medium serving</td>
<td>17</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Ice cream</td>
<td>1 scoop or 1/2 cup</td>
<td>84</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Yogurt, frozen yogurt</td>
<td>1 cup</td>
<td>212</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Milk on cereal</td>
<td>1/2 cup</td>
<td>175</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Oysters</td>
<td>6 medium</td>
<td>24</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Shrimp</td>
<td>3 oz</td>
<td>33</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Pink salmon</td>
<td>3 oz</td>
<td>181</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Tuna, tuna salad, tuna casserole</td>
<td>1/2 cup</td>
<td>12</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>Liver, including chicken livers</td>
<td>4 oz</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Eggs</td>
<td>1 medium egg, small 2 eggs</td>
<td>23</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>High fiber, bran or granola cereals, shredded wheat</td>
<td>1 medium bowl</td>
<td>88</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Participant I.D. #</td>
<td>Start of Study Calcium (mg/d)</td>
<td>Vitamin D (IU/d)</td>
<td>End of Study Calcium (mg/d)</td>
<td>Vitamin D (IU/d)</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------------------------------</td>
<td>-----------------</td>
<td>----------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>002</td>
<td>324.04</td>
<td>75.25</td>
<td>548.00</td>
<td>114.33</td>
</tr>
<tr>
<td>004</td>
<td>452.48</td>
<td>78.46</td>
<td>Withdrawal</td>
<td></td>
</tr>
<tr>
<td>005</td>
<td>466.56</td>
<td>75.79</td>
<td>363.69</td>
<td>75.25</td>
</tr>
<tr>
<td>006</td>
<td>103.31</td>
<td>44.17</td>
<td>103.31</td>
<td>44.17</td>
</tr>
<tr>
<td>007</td>
<td>218.65</td>
<td>48.25</td>
<td>Withdrawal</td>
<td></td>
</tr>
<tr>
<td>008</td>
<td>607.83</td>
<td>107.08</td>
<td>721.17</td>
<td>125.58</td>
</tr>
<tr>
<td>009</td>
<td>1313.10</td>
<td>334.76</td>
<td>1257.04</td>
<td>278.38</td>
</tr>
<tr>
<td>012</td>
<td>1048.33</td>
<td>274.67</td>
<td>547.69</td>
<td>133.58</td>
</tr>
<tr>
<td>013</td>
<td>804.98</td>
<td>131.71</td>
<td>1051.83</td>
<td>194.00</td>
</tr>
<tr>
<td>015</td>
<td>274.83</td>
<td>27.25</td>
<td>87.17</td>
<td>8.58</td>
</tr>
<tr>
<td>016</td>
<td>159.04</td>
<td>37.65</td>
<td>173.40</td>
<td>43.00</td>
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<td>176.35</td>
<td>755.44</td>
<td>197.58</td>
</tr>
<tr>
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<td>770.58</td>
<td>56.00</td>
<td>763.13</td>
<td>101.25</td>
</tr>
<tr>
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<td>1062.42</td>
<td>191.83</td>
<td>1062.42</td>
<td>191.83</td>
</tr>
<tr>
<td>024</td>
<td>647.17</td>
<td>119.33</td>
<td>379.50</td>
<td>78.83</td>
</tr>
<tr>
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<td>212.58</td>
<td>43.88</td>
<td>138.19</td>
<td>19.83</td>
</tr>
<tr>
<td>028</td>
<td>655.75</td>
<td>148.88</td>
<td>382.08</td>
<td>61.25</td>
</tr>
<tr>
<td>029</td>
<td>1413.15</td>
<td>280.23</td>
<td>1199.83</td>
<td>272.58</td>
</tr>
<tr>
<td><strong>Average Intake</strong></td>
<td><strong>623.06 ± 392.28</strong></td>
<td><strong>125.08 ± 92.85</strong></td>
<td><strong>595.87 ± 394.71</strong></td>
<td><strong>121.25 ± 84.40</strong></td>
</tr>
</tbody>
</table>

4.3.1 Bioavailability of Vitamin D in an Aqueous Solution

At the beginning of the study, according to the Institute of Medicine (IOM) 1 (5.5%) individual had a low level of vitamin D (27.61 nmol/L) which is generally inadequate for bone and overall health in a healthy individual. A slight reduction in serum would have rendered this individual to be classified as vitamin D deficient if the serum level was ≤ 25.0 nmol/L. Thirteen (72.2%) study participants were considered to have adequate vitamin D status, according to the
IOM a serum concentration of ≥37.5 nmol/L (≥15 ng/mL) constitutes adequate vitamin D, which is considered adequate for bone and overall health according to the current standards. The normal range is typically 37.5 – 80.0 nmol/L, as seen by commercial assays (IOM, 1997). On the other hand, according to the proposed adjustments in serum vitamin D classification these same individuals would be considered vitamin D insufficient (Holick, 2005). These individuals would have adequate vitamin D stores to maintain healthy bones and teeth, but may not have the appropriate amount of vitamin D to aid in the prevention of development some cancers and other chronic diseases. Three (16.6%) of the individuals at the start of the study were considered to have optimal serum vitamin D levels, serum levels 80-120 nmol/L. Only one person in the optimal serum vitamin D level category consumed a daily multi-vitamin containing vitamin D. After the duration of the study, 10 (55.5%) participants had adequate vitamin D serum level, whereas 8 (44.4%) subjects were considered to have an optimal vitamin D level. There was no significant correlation between the average vitamin D intake determine by the two food frequency questionnaires and the average baseline serum vitamin D concentration in the 16 subjects.

The mean distribution of each treatment group and control is illustrated in Table 4.4. Due to trial modifications treatments 400 IU vitamin D (control, 400 IU D₂, and 400 IU D₃) and the remaining treatments (control, 600 IU D₂, 600 IU D₃, 1000 IU D₂, and 1000 IU D₃) were evaluated separately.

As illustrated in Figure 4.1, for the 400 IU vitamin D trials baseline serum characteristics were similar in all groups. Significant effects (p =0.0144) of time between 0 and 24 hours revealed a change in serum vitamin D levels. Moreover, there was a significant difference
(p=0.0450) between the control compared to the intervention groups. There was no significant difference between vitamin D$_2$ and vitamin D$_3$ intervention groups.

When analyses of the remaining treatments were conducted, there were no significant effects of treatment type, gender, time, or age. However, there was a significant difference as it related to race (p<0.0001), as shown in Table 4.5 – Table 4.7 and Figure 4.21 – Figure 4.22. Serum vitamin D levels were significantly lower in minorities compared to Caucasians at each time point. Figure 4.2 is a visual representation of the average means serum vitamin D levels for each treatment.

A significant difference was seen at from initial baseline (first treatment) to the initial baseline (last treatment) for each individual who completed more than one treatment. Table 4.6 illustrates the mean change in serum vitamin D throughout the study. There was an average increase of 54 ± 27.1 nmol/L units of serum vitamin D for each individual. Figures 4.3 thru 4.20 illustrate the change in serum vitamin D level during all completed trials for each individual.

### 4.4 Discussion and Conclusion

The current study is, to our knowledge, the first randomized controlled trial that involved a vitamin D intervention with a fortified vitamin D flavored aqueous beverage. First, this portion of the study had several limitations. The small sample size was not a true representation of the US population. Moreover, serum calcium status should have been investigated to determine if appropriate levels were present at the start of the study. There was no positive control to illustrate a true effect of vitamin D in the beverages on serum levels. With these limitations, notably there was no significant difference in vitamin D status among individuals who consumed a multi-vitamin containing vitamin D versus those who did not.
Additionally, no difference in vitamin D status was seen among gender lines. Previous research has indicated that among the United States’ population, women’s mean intake of vitamin D may be lower than their male counterparts (Moore et al., 2004). Investigation was completed on the self-reported modified food frequency questionnaire, data were extrapolated, and it was observed that daily calcium and vitamin D was below RDA in the majority of participants.

These findings are in concert with previous data that have shown vitamin intake below RDA (Moore et al., 2004). However, extrapolation of the diet via FFQ may have not truly assessed the calcium and vitamin D statuses of the subjects; serum calcium along with vitamin D status could have been used as additional biomarkers of intake. FFQ assessed the participants recalled food intake over an extended period of time. Research studies have compared FFQ versus other methods of collecting food intake such as, diet recall/record in predicting vitamin D intake. These studies have found a greater correlation between vitamin D status and reported intake via FFQ than other methods (Blalock et al., 2003, Taylor et al., 2008, Wu et al., 2009).

Nevertheless, it should be noted that even though initial calcium and vitamin D intake decreased during the study in the majority of the participants according to the FFQ data, serum vitamin D levels increased in all participants between their first and last visit. These results may indicate that consumption of the vitamin D-fortified beverage had a positive effect on serum 25(OH) vitamin D levels.

Our results show that vitamin D is bioavailable from a fortified beta-lactoglobulin-vitamin D aqueous drink. This study utilized the gold standard of determination of vitamin D status, serum 25(OH) vitamin D (Holick, 2005). The change in 25(OH) vitamin D was seen from the initial to the finale treatment. A few studies have reported that daily consumption of a
fortified food or beverage fortified with vitamin D can increase serum 25(OH) vitamin D levels (Wagner et al., 2008 and Tangpricha et al., 2003). Moreover, these studies illustrated that milk is not the only food item that can effectively be fortified with vitamin D. Several studies investigated fortification of cheese and orange juice as vehicles for fortification (Wagner et al., 2008; Tangpricha et al., 2003). Furthermore, the fortified aqueous beverage produced a change in serum 25(OH) vitamin D that did not differ due to vitamer source (D_2 versus D_3). These findings are different from previous research that has stated that D_3 is a more effective fortifier than D_2 (Armas et al., 2004). On the other hand, both studies (Wagner et al., 2008 and Tangpricha et al., 2003) investigated vitamin D fortification to higher levels than the current vitamin D fortification recommendations. Other research studies have investigated vitamin D supplementation ranging from 400 IU to 10,000 IU of vitamin D and have found a positive effect in reduction of falls, fractures and increase in serum 25-OH vitamin D with no adverse health effects (Holick, 1999 and Trivedi et al., 2003). As illustrated by this study, there was no significant difference in hourly serum 25(OH) vitamin D after ingesting the beverage. This was in contrast with previous studies, which have shown significant hourly time effects with high doses of vitamin D (Tangpricha et al., 2008) during the time period used in this study (Johnson et al., 2005). This may have been due to greater hourly evaluation time, baseline to 72 hours post-consumption. In this study, significant increase in serum vitamin D was seen from the beginning to end of the study, similar to previous studies (Johnson et al., 2005, Tangpricha et al., 2008), indicating linearly increasing vitamin D levels over time of supplementation. It should also be noted, that some individuals’ control serum values were higher than subsequent interventions. This may be due to the randomization process, which allows for control at any
time point, and a carry-over effect from an earlier day’s treatment. Levels of vitamin D may have already been increased by consuming the vitamin D fortified beverages.

Our results could not demonstrated that vitamin D-fortified aqueous beverages are indeed bioavailable, because we did not see the expected dose response in serum at between 600 and 1000 IU, and because individuals did not consistently have a transient rise in serum vitamin D after an oral dose. The inability to measure area under the curve with treatment may have prevented seeing potential differences among the fortified beverages following a single oral dose as seen in previous studies that used much higher test doses, such Johnston et al., (2005).

Additionally, the research illustrated that beverages containing no fat could be fortified with vitamin D. Tangpricha et al. (2003) showed similar results; fat content did not affect vitamin D bioavailability between whole milk, skim milk, corn oil on toast, or fortified orange juice. It was concluded from this study that ingestion of a beta-lactoglobulin vitamin D complex beverage did not prevent changes in serum 25(OH) vitamin D in some subjects indicating that vitamin D might be absorbed from a non-fat aqueous drink.

Previous studies have shown that there are several minority groups who are at greater risk of vitamin D insufficiency (Calvo et al., 2006). Results from this study demonstrated that minorities have lower serum 25(OH) vitamin D levels. Moreover, these individuals (African Americans and Asians) may have been at greater risk of vitamin D insufficiency due to several factors: darkly pigmented skin, whose cutaneous vitamin D synthesis is low because of competition of melanin; higher rates of lactose intolerance that may limit intake of fortified milk; and cultural diets that are low in cows’ milk, which is one of the main fortified food sources of vitamin D, due to consumption prevalence of soy in the diet (Calvo et al., 2006). These barriers
suggest the necessity to improve vitamin D status among minorities and investigate alternative vehicles of vitamin D fortification.

In conclusion, suboptimal vitamin D status is common in the United States. Fortification of non-traditional food items (other than milk and ready-to-eat cereals) may be an effective approach to improve vitamin D status. Moreover, as shown by this research a water soluble form of vitamin D can be used to fortify aqueous products to improve vitamin D intake. In this study, fortifying with vitamin D$_2$ or vitamin D$_3$ obtained from the same supplier did not affect the serum 25(OH) vitamin D differently.
Table 4.4 Mean Distribution: Serum 25(OH)D (nmol/L)

<table>
<thead>
<tr>
<th>level</th>
<th>d</th>
<th>time</th>
<th>N</th>
<th>Obs</th>
<th>N</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Minimum</th>
<th>Maximum</th>
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</thead>
<tbody>
<tr>
<td>400</td>
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<td>2</td>
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<td>16</td>
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<td>16</td>
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<td>17</td>
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<td>3</td>
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<td>17</td>
<td>74.27</td>
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<td>4</td>
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<td>17</td>
<td>68.04</td>
<td>28.42</td>
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<td>16</td>
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<tr>
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<td>16</td>
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<tr>
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<td>4</td>
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<td>16</td>
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<td>55.77</td>
<td>16.42</td>
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</table>
Table 4.5 GLM Procedure: Repeated Measures Analysis of Variance Test of Hypotheses for Between Subject Effect. Level = quantity of vitamin D in the oral dose. D = form of vitamin D (D$_2$ or D$_3$) in the beverage.

<table>
<thead>
<tr>
<th>Source</th>
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<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
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</tr>
<tr>
<td>d(control)</td>
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<td>level*d(control)</td>
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Table 4.6 Change in Mean Vitamin D Status

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<tr>
<td>April 27-30</td>
<td>69.28±29.45</td>
<td>68.46±29.94</td>
<td>59.85±28.16</td>
<td>66.67±30.41</td>
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<td>0hr</td>
<td>4hr</td>
<td>8hr</td>
<td>24hr</td>
</tr>
<tr>
<td>May 18-21</td>
<td>73.25±32.53</td>
<td>70.93±35.81</td>
<td>68.19±28.57</td>
<td>68.49±36.65</td>
</tr>
<tr>
<td>June 15-18</td>
<td>65.18±33.06</td>
<td>49.89±19.25</td>
<td>56.41±29.38</td>
<td>53.44±25.32</td>
</tr>
<tr>
<td>July 6-9</td>
<td>78.65±42.01</td>
<td>74.31±42.59</td>
<td>73.11±44.90</td>
<td>75.16±46.99</td>
</tr>
<tr>
<td>July 27-30</td>
<td>98.33±55.48</td>
<td>104.99±74.92</td>
<td>105.65±56.37</td>
<td>97.23±62.13</td>
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### Table 4.7 Comparison of Mean Serum 25(OH)D Between Ethnic Groups

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Serum 25(OH)D (nmol/L) (mean±S.D.)</th>
<th>Caucasian (n=13)</th>
<th>Minorities (n=5)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Time 0 4 8 24</td>
<td>0 4 8 24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control 100.87 ± 55.39</td>
<td>100.87 ± 55.39</td>
<td>25.94 ± 9.31</td>
</tr>
<tr>
<td></td>
<td>1000 IU D2 94.27 ± 32.29</td>
<td>94.27 ± 32.29</td>
<td>31.23 ± 7.25</td>
</tr>
<tr>
<td></td>
<td>1000 IU D3 93.12 ± 40.8</td>
<td>93.12 ± 40.8</td>
<td>35.32 ± 13.72</td>
</tr>
<tr>
<td></td>
<td>600 IU D2 77.92 ± 11.28</td>
<td>77.92 ± 11.28</td>
<td>36.47 ± 6.13</td>
</tr>
<tr>
<td></td>
<td>600 IU D3 93.82 ± 34.33</td>
<td>93.82 ± 34.33</td>
<td>39.21 ± 14.99</td>
</tr>
<tr>
<td></td>
<td>1000 IU (D2&amp;D3) 93.70 ± 35.99</td>
<td>93.70 ± 35.99</td>
<td>39.50 ± 14.03</td>
</tr>
<tr>
<td></td>
<td>600 IU (D2&amp;D3) 86.60 ± 26.63</td>
<td>86.60 ± 26.63</td>
<td>35.79 ± 5.50</td>
</tr>
</tbody>
</table>

NA- Not applicable
Figure 4.1 Mean Serum 25(OH) vitamin D Concentration in Subjects consuming Control and Fortified Aqueous 400 IU Vitamin D Beverages (D$_2$ or D$_3$)
Figure 4.2 Mean Serum 25(OH) vitamin D Concentration in Subjects Consuming Control, 600 IU and 1000 IU Fortified Aqueous Vitamin D (D₂ and D₃)
Figure 4.3 Participant 002: Serum 25(OH)D
Figure 4.4 Participant 004: Serum 25(OH)D
Figure 4.5 Participate 005: Serum 25(OH)D
Figure 4.6 Participant 006: Serum 25(OH)D
Figure 4.7 Participant 007: Serum 25(OH)D
Figure 4.8 Participant 008: Serum 25(OH)D
Figure 4.9 Participant 009: Serum 25(OH)D
Figure 4.10 Participant 012: Serum 25(OH)D
Figure 4.11 Participant 013: Serum 25(OH)D
Figure 4.12 Participant 015: Serum 25(OH)D
Figure 4.13 Participant 016: Serum 25(OH)D
Figure 4.14 Participant 017: Serum 25(OH)D
Figure 4.15 Participant 018: Serum 25(OH)D
Figure 4.16 Participant 019: Serum 25(OH)D
Figure 4.17 Participant 024: Serum 25(OH)D
Figure 4.18 Participant 027: Serum 25(OH)D
Figure 4.19 Participant 028: Serum 25(OH)D
Figure 4.20 Participant 029: Serum 25(OH)D
Figure 4.21 Mean Serum 25(OH)D Comparison between Ethnic Groups: Vitamer Type
Figure 4.22 Mean Serum 25(OH)D Comparison by Ethnic Groups: Amount of Vitamin D
REFERENCES


CHAPTER 5

Summary and Conclusion

Mallorye D. Lovett

Department of Food, Bioprocessing and Nutrition Sciences

North Carolina State University
5.1 Summary and Conclusion

Vitamin D is an essential hormone that aids in the absorption of calcium and phosphorus. Vitamin D has a pivotal role in skeletal health (Maalouf et al., 2008). The primary source of dietary vitamin D is fortified dairy products, mainly fluid milk. Moreover, vitamin D is also derived naturally from cutaneous synthesis when skin is exposed to ultraviolet radiation from sunlight (Holick, 1992 and Holick, 2005). The reduction in dietary consumption of vitamin D, under-fortification of vitamins in fluid milk, and decreased exposure to sunlight has lead to suboptimal vitamin D status globally (Calvo et al., 2005, Holick, 1999, and Murphy, 2001). Vitamin D has been shown to be bioavailable in milk, orange juice, and cheddar cheese (Tangpricha et al., 2003 and Wagner et al., 2008). This research studied the potential of β-lactoglobulin as a soluble and stable carrier of vitamin D in an aqueous system via analytical testing and a human bioavailability study.

To develop the protein-based fortifiers (Chapter 3), β-LG vitamin D complexes were prepared in a molar ratio of 1:1 based on previous research indicating the binding characteristics of β-LG with vitamin A and D (Wang et al., 1999). Fluorescence spectroscopy was conducted on β-LG vitamin D complexes to demonstrate binding capacity of the protein for vitamin D supplied in a powder suspension. Moreover, binding capacity utilizing fluorescence spectroscopy was performed on both spray-dried complexes and non spray-dried complexes. On a small scale powder recovery and vitamin D contents was suboptimal. Spray-drying has been widely utilized as an effective tool in the food and pharmaceutical industry (Ameri, 2006). Additional research should focus on optimization of spray drying protocol, which would help translate the small scale production of β-LG vitamin D complexes to a larger scale.
Increasing interest in developing systems for protein vitamin complexes had lead to the proposal that spray-drying may provide the means to accomplish this process. Spray drying is one of the primary procedures utilized to generate a dried powder. Spray drying transforms liquid solutions into dried powders by rapidly exposing the liquid solution to hot, dry gases (Ameri et al., 2006). Moreover, to achieve long-term stability of protein vitamin complexes researchers have investigate the use of various proteins to increase binding and various sugars to protect the proteins during spray drying (Liao et al., 2002). This study utilized \( \beta-LG \) as the protein source and investigated lactose versus trehalose on the preservation of the native protein. The preservation capacity of one sugar over the other appeared to be equivocal based on the binding capacity of vitamin D to the protein. The majority of research studies investigating spray drying of proteins has utilized small scale spray dryers and has not investigated the stabilizing affect of various sugars on a protein. Additional research is needed to fully understand the protective effect of disaccharides on protein, more specially beta-lactoglobulin.

HPLC has been the gold standard for determination of vitamin D. The HPLC method has quantified vitamin D in various media including serum and food products. However, when utilizing the HPLC method there are some disadvantages that researchers may encounter including 1) highly skilled analyst required, 2) higher cost per sample analyzed, and 3) variable reproducibility. This study examined the difference in vitamin D content when comparing the gold standard, HPLC, to a new analysis kit, VitaKit D™. No appreciable differences were seen between the two methods when investigating vitamin D content of a vitamin D fortified beverage. However, VitaKit D™ can only determine total vitamin D content, whereas HPLC is
able to determine a range of vitamin D metabolites. Future research should be conducted to determine if VitaKit D™ is as reliable as HPLC to determine vitamin D content in food products.

Bioavailability of beta-lactoglobulin vitamin D complexes were investigated using a human intervention study. Results from serum 25(OH) vitamin D ELISA assay was used as an indicator for vitamin D bioavailability. Participants consumed an assortment of β-LG-vitamin D complex beverages including a control and beverages containing increasing amounts of vitamin D (400 IU, 600 IU, 1000 IU) with different vitamer sources (D$_2$ or D$_3$). Unfortunately, the expected transient increases in serum vitamin D after the oral dosage of the fortified beverages could not be observed. More research is needed to develop methods to measure bioavailability of the dosages of D vitamers commonly found in foods.

These studies illustrated that a fortified β-LG-vitamin D aqueous beverage could adequately be fortified with vitamin D and contain similar amounts of vitamin D compared to milk samples (Chapter 3). Increasing vitamin D intake (600 IU and 1000 IU) showed no significant effect on serum vitamin D immediately after intake. Moreover, there was no significant correlation between the usual vitamin D intake determined by food frequency questionnaire and baseline vitamin D concentration in serum. Significant differences in serum vitamin D levels were seen among race/ethnicity groups; minorities had lower serum vitamin D levels overall which continues to highlight the need for research to address health status disparities among minorities (Moore et al., 2005). Overall the study illustrated that baseline serum levels were significantly different from the beginning to the completion of the study, after oral doses of the fortified beverage had been given at 2-week intervals. Because the study
spanned the months April to July, it appears that increasing intensity of sunlight had greater impact on average serum 25-OH than did dietary intake. Additionally, this study identified the need, similar to other research studies, to investigate the current vitamin D recommendation (Calvo et al., 2005 and Holick, 2005). From the review of the results it is apparent that current estimates of vitamin D intake are below the current recommendations, and these results illustrate that further research must be conducted to examine that adequacy of current recommended intakes to address the issues of suboptimal vitamin D status as it relates to vitamin D insufficiency. It is incumbent that continued research must address vitamin D insufficiency in a multicultural, multi-gendered and multi-demographic population. Furthermore, future research should address the efficacy and safety of new fortification strategies for vitamin D.

In conclusion, β-LG-vitamin D complex is an appropriate fortifier for an aqueous drink. The β-LG-vitamin D complex fortified beverage had the ability to increase serum vitamin D levels. This study highlights the importance of fortification as an avenue to achieve optimum vitamin D status.
REFERENCES


Appendix A

Vitamin D Intervention Study - Recruitment

Two researchers (Jonathan C. Allen, PhD, CNS and Mallorye Lovett, MS, RD, LDN) at North Carolina State University in the Department of Food, Bioprocessing, and Nutrition Sciences are conducting a five-month research study on the effect of vitamin D supplementation in a water beverage, on blood levels. This research study will take place in Schaub Hall on NCSU’s main campus.

Subjects will have to consume the beverage on five (5) separate occasions with two week intervals between samples. Subjects will collect their own blood via finger prick, 4 times per treatment. Subjects will be financially compensated for their participation; subjects will receive a $20 gift cards at each treatment time.

If you may be interested in participating please complete the brief anonymous survey to see if you are eligible for participation. Please provide accurate answers to the survey. This survey is anonymous; subjects will only provide contact information if qualified. Additionally, the survey is hosted via a public URL therefore IP addresses may be collected but they will not be associated with responses. If you have questions, please call Mallorye Lovett at (919) 513-2278 or contact via e-mail mdlovett@ncsu.edu.

Thank you,
Mallorye Lovett
### Appendix B

**ANONYMOUS Computerized Questionnaire to Determine Eligibility**

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<tr>
<th>Question</th>
<th>Answer</th>
<th>Score</th>
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</thead>
</table>
| Are you between the age of 18-50 (years)?                                | YES    | NO  
|                                                                           |        | Yes- STOP survey - individual does not qualify                                              |
| Have you been diagnosed with chronic liver disease?                      | YES    | NO  
|                                                                           |        | Yes- STOP survey - individual does not qualify                                              |
| Have you been diagnosed with kidney disease?                             | YES    | NO  
|                                                                           |        | Yes- STOP survey - individual does not qualify                                              |
| Are you taking medications for liver or kidney disease?                  | YES    | NO  
|                                                                           |        | Yes- STOP survey – individual does not qualify                                              |
| Do you have a family history of kidney or liver disease?                 | YES    | NO  
|                                                                           |        | Yes – STOP survey – individual does not qualify                                              |
| Are you trying to become pregnant, or think you may be pregnant?         | YES    | NO  
|                                                                           |        | Yes- STOP survey – individual does not qualify                                              |
| Have you been diagnosed with a lactose (found in milk) allergy?          | YES    | NO  
|                                                                           |        | Yes- STOP survey – individual does not qualify                                              |
| Have you been diagnosed with lactose intolerance?                        | YES    | NO  
<p>|                                                                           |        | No – continue to next question                                                             |</p>
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<thead>
<tr>
<th>Question</th>
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<td>Do you take any anticonvulsants medications? (i.e. Depakote, Depakene (divalproex sodium, valproic acid, or valproate sodium); Tegretol (carbamazepine); Lamicatal (lamotrigine); Trileptal (oxcarbazepine))</td>
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<td>NO</td>
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<tr>
<td>Do you take any barbiturates? (i.e. Amobarbital (downers, blue heavens); Pentobarbital (nembies, yellow jackets); Phenobarbital (purple hearts, goof balls); Secobarbital (reds, red birds, red devils); Tuinal (rainbows, reds and blues, tooties))</td>
<td>YES</td>
<td>NO</td>
<td>Yes- STOP survey - individual does not qualify</td>
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<tr>
<td>Do you take medications in the morning that must be consumed with food?</td>
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<tr>
<td>Do you take calcium supplements?</td>
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<tr>
<td>Do you take Tums™?</td>
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<tr>
<td>Do you take vitamin D supplements?</td>
<td>YES</td>
<td>NO</td>
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<td>Do you take a multivitamin?</td>
<td>YES</td>
<td>NO</td>
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**Demographic Information**

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<tr>
<td>Asian</td>
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<tr>
<td>American Indian or Alaska Native</td>
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<tr>
<td>Other</td>
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</table>
Appendix C

Consent Form

IRB Study #: 

Title of Study: Using a Whey Protein to Appropriately Fortify Vitamin D in Aqueous Beverages

Principal Investigator: Mallorye Lovett

NC State University Department: Food, Bioprocessing, and Nutrition Sciences

Faculty Advisor: Jonathan C. Allen, PhD

Study Contact telephone number: 919-513-2278
Study contact e-mail: mdlovett@ncsu.edu

What should you know about research studies:
You are being asked to participate in a research study. To join the study is voluntary. You may refuse to join, or you may withdraw your consent to be in the study, for any reason. Deciding not to be in the study or leaving the study before it is done will not affect your relationship with the researcher or North Carolina State University.

Research studies are designed to obtain new knowledge that may help other people in the future.

Details about this study are discussed below. It is important that you understand this information so that you can make an informed choice about being in this research study. You will be given a copy of this consent form. You should ask the researchers named above or faculty advisor, any questions you have about the study at any time.

Purpose of the Study:
It has been well established that people in the United States do not get enough vitamin D. Poor vitamin D status has been correlated to increased prevalence of chronic disease. The purpose of this study is to investigate the effect of a water-soluble form of vitamin D in a sports drink beverage when consumed. The researchers are investigating if this new formulation will be more easily absorbed by the body. In addition, the researchers are comparing absorption of vitamin D₂ versus D₃ in a water soluble form.

Reasons not to participate in study:
1. Individuals with liver and kidney diseases or a family history of these diseases, those taking medications (i.e. anticonvulsants, barbiturates, and illicit drugs) that may affect vitamin D metabolism and subjects who are taking vitamin D supplement will be excluded from the trial.
2. Individuals taking medication for liver or kidney disease.
3. Subjects will be asked to restrict exposure to sunlight for the 24 hour period and complete a food frequency questionnaire which will be analyzed.
4. If you are not willing to have your blood drawn by finger prick, you should not participate in the study.
5. If you take any medications that require food to be taken with them, you should not participate in this study.
6. If you take TUMS® or another antacid containing calcium.
7. If you are trying to become pregnant, or think you may be pregnant, you should refrain from participating.
8. In addition, if you have a milk allergy or lactose intolerance.

Length of study: If you decide to be in this study, you will participate in five (5) treatment sessions. At each treatment session you will consume 8 fluid ounces of a vitamin D (D2 or D3) fortified beverage. The beverages will be fortified with 400 IU, 600 IU, or 1000 IU of vitamin D. Blood samples will be collected fasting, 6, 12, and 24 hours post-consumption. Each treatment may take approximately four (4) hours of your time during each treatment session. You will be asked to prick your finger a total of 20 times over a 14 week period. You will consume 4000 IU (not to exceed) of vitamin D in a 14 week period. You'll also be asked to complete a food frequency questionnaire at the start and completion of the study. Please see the attached table of events for detailed information about your participation.

Procedure of study: 1. Complete anonymous online eligibility survey.
2. If eligible, participants will receive study schedule and copy of consent form via e-mail.
3. Avoid direct sunlight for 24 hours at each point of intervention.
4. Arrive at NC State University Schaub Hall 216 from 8:00 am – 8:30 am on day of intervention. First day of study sign consent form in front of researcher.
5. Collect blood via finger prick upon arrival.
6. Consume 8 fl. oz of vitamin D fortified beverage within 30 minutes.
7. Return to lab to collect blood at 6, 12, 24 hours post consumption.
8. Two week break period between interventions.

Possible risk or discomforts:
There may be discomfort associated with collection of blood samples. Discomfort may consist of soreness at the site where you prick your finger. No other analysis including drug screening, HIV testing will be done on your blood. All samples will be destroyed after they are tested for the purposes of the study.

There should be no risk to you from the vitamin D treatments. The highest amount of vitamin D that you will receive per treatment is 1000 IU (international units). The maximum daily intake for vitamin D has been recommended as 2,000 IU/day. Vitamin D levels within the range of
Adequate Intake (AI) thru Upper Level (UL) have not been shown to have any adverse health effects. (IOM, 2003)

**Benefits:**
It is unlikely you will gain any personal benefit from consuming the vitamin D fortified beverage as it relates to serum vitamin D levels. However, this study is meant to answer scientific questions about vitamin D fortification in an aqueous solution and its effect on vitamin D status. The results may give investigators information to use in future research and increase the body of research regarding vitamin D fortification.

**Privacy Protection:**
No subjects will be identified in any report or publication about this study. Personal information will only be viewed by researcher and faculty advisor.

**Discontinue Participation:**
You can withdraw from this study at any time, without penalty or loss of benefits to which you are otherwise entitled to. The investigators also have the right to stop your participation at any time. This could be because you have had an unexpected reaction, or have failed to follow instructions, or because the entire study has been discontinued.

**Compensation:**
You will receive a $20 dollar gift card at the end of each of the five treatment sessions.

**Emergency Medical Treatment:**
During the duration of the study, subjects will ingest a fortified beverage. The consumption of this beverage will cause minimal risk because all ingredients are commercially available for human consumption. Subjects will collect blood samples via finger pricks to determine the effectiveness of the fortified beverage. At all times during participation, subjects will have access to first aid supplies (gauze, band-aid, etc.) and fluids (water and soda). For additional medical concerns subjects will be directed to their own physician for further medical treatment.

**North Carolina State Students:**
You may choose not to be in the study or to stop being in the study before it is over at any time. This will not affect your class standing or grades at North Carolina State University. You will not be offered or receive any special consideration if you take part in this research.

**Questions about study:**
You have the right to ask, and have answered, any questions you may have about this research. If you have questions, or if a research-related injury occurs, you should contact the researchers listed on the first page of this form.

**Rights as a research subject:**
All research on human volunteers is reviewed by a committee that works to protect your rights and welfare. If you have questions or concerns about your rights as a research subject you may
contact the Institutional Review Board. If you feel you have not been treated according to the descriptions in this form, or your rights as a participant in research have been violated during the course of this project, you may contact Deb Paxton, Regulatory Compliance Administrator, Box 7514, NCSU Campus (919/515-4514), or Joe Rabiega, IRB Coordinator, Box 7514, NCSU Campus (919/515-7515).

Study Schedule:

<table>
<thead>
<tr>
<th>Study Schedule</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Treatment Day #1</strong>&lt;br&gt;March 9-12, 2009</td>
<td>Subjects will consume a fortified vitamin D beverage that has been prepared with commercial ingredients and produced in a commercial dairy facility at NCSU. The beverage is cold-filled within a sterile can and stored at 4C no more than 7 days.</td>
</tr>
<tr>
<td>Fast after midnight</td>
<td>Please take essential medications with a drink of water.</td>
</tr>
<tr>
<td>8:00 AM</td>
<td>Arrive at lab (Schaub Hall 216), complete survey, finger prick, and drink 8 fluid ounce beverage. The beverage will contain either no vitamin D, 400 IU D2, or 400 IU D3 (randomly assigned).</td>
</tr>
<tr>
<td>2:00 PM</td>
<td>Return to lab for finger prick</td>
</tr>
<tr>
<td>8:00 PM</td>
<td>Return to lab for finger prick</td>
</tr>
<tr>
<td>8:00 AM next morning</td>
<td>Two week period before next treatment</td>
</tr>
<tr>
<td><strong>Treatment Day #2</strong>&lt;br&gt;March 23-26, 2009</td>
<td>Subjects will consume a fortified vitamin D beverage that has been prepared with commercial ingredients and produced in a commercial dairy facility at NCSU. The beverage is cold-filled within a sterile can and stored at 4C no more than 7 days.</td>
</tr>
<tr>
<td>Fast after midnight</td>
<td>Please take essential medications with a drink of water.</td>
</tr>
<tr>
<td>8:00 AM</td>
<td>Arrive at lab (Schaub Hall 216), complete survey, finger prick, and drink 8 fluid ounce beverage. The beverage will contain either no vitamin D, 400 IU D2, or 400 IU D3 (randomly assigned).</td>
</tr>
<tr>
<td>2:00 PM</td>
<td>Return to lab for finger prick</td>
</tr>
<tr>
<td>8:00 PM</td>
<td>Return to lab for finger prick</td>
</tr>
<tr>
<td>8:00 AM next morning</td>
<td>Return to lab for finger prick</td>
</tr>
<tr>
<td>Two week period before next treatment</td>
<td></td>
</tr>
<tr>
<td><strong>Treatment Day #3</strong>&lt;br&gt;April 13-16, 2009</td>
<td>Subjects will consume a fortified vitamin D beverage that has been prepared with commercial ingredients and produced in a commercial dairy facility at NCSU. The beverage is cold-filled within a sterile can and stored at 4C no more than 7 days.</td>
</tr>
<tr>
<td>Fast after midnight</td>
<td>Please take essential medications with a drink of water.</td>
</tr>
<tr>
<td>Time</td>
<td>Activity</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>8:00 AM</td>
<td>Arrive at lab (Schaub Hall 216), complete survey, finger prick, and drink 8 fluid ounce beverage. The beverage will contain either no vitamin D, 400 IU D$_2$, or 400 IU D$_3$ (randomly assigned).</td>
</tr>
<tr>
<td>2:00 PM</td>
<td>Return to lab for finger prick</td>
</tr>
<tr>
<td>8:00 PM</td>
<td>Return to lab for finger prick</td>
</tr>
<tr>
<td>8:00 AM next morning</td>
<td>Return to lab for finger prick</td>
</tr>
<tr>
<td></td>
<td>Two week period before next treatment</td>
</tr>
</tbody>
</table>

**Treatment Day #4**  
*May 4-7, 2009*

Subjects will consume a fortified vitamin D beverage that has been prepared with commercial ingredients and produced in a commercial dairy facility at NCSU. The beverage is cold-filled within a sterile can and stored at 4C no more than 7 days.

- **Fast after midnight**: Please take essential medications with a drink of water.
- 8:00 AM  
  Arrive at lab (Schaub Hall 216), complete survey, finger prick, and drink 8 fluid ounce beverage. The fortified beverage will contain 400 IU, 600 IU, or 1000 IU of vitamin D (randomly assigned).
- 2:00 PM  
  Return to lab for finger prick
- 8:00 PM  
  Return to lab for finger prick
- 8:00 AM next morning  
  Return to lab for finger prick
- Two week period before next treatment

**Treatment Day #5**  
*May 25-21, 2009*

Subjects will consume a fortified vitamin D beverage that has been prepared with commercial ingredients and produced in a commercial dairy facility at NCSU. The beverage is cold-filled within a sterile can and stored at 4C no more than 7 days.

- **Fast after midnight**: Please take essential medications with a drink of water.
- 8:00 AM  
  Arrive at lab (Schaub Hall 216), complete survey, finger prick, and drink 8 fluid ounce beverage. The fortified beverage will contain 400 IU, 600 IU, or 1000 IU of vitamin D (randomly assigned).
- 2:00 PM  
  Return to lab for finger prick
- 8:00 PM  
  Return to lab for finger prick
- 8:00 AM next morning  
  Return to lab for finger prick
- Two week period before next treatment

**Thank for your participation**
Title of Study: Using a Whey Protein to Appropriately Fortify Vitamin D in Aqueous Beverages

Principal Investigator: Mallorye Lovett

Subject’s Agreement:
I have read the information provided above. I have asked all the questions I have at this time. I voluntarily agree to participate in this research study.

______________________________  __________________________
Signature of Research Subject  Date

______________________________
Printed Name of Research Subject

______________________________  __________________________
Signature of Person Obtaining Consent  Date

______________________________
Printed Name of Person Obtaining Consent