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Comparative analysis of vitamin D content in sardines canned in olive oil and water

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COMPARATIVE ANALYSIS OF VITAMIN D CONTENT IN SARDINES CANNED IN OLIVE OIL AND WATER

by

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DEDICATION

This thesis is dedicated to my family for their unconditional support and guidance.

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COMPARATIVE ANALYSIS OF VITAMIN D CONTENT

IN SARDINES CANNED IN OLIVE OIL AND WATER

TYLER KALAJIAN

ABSTRACT

Vitamin D is a fat-soluble hormone primarily responsible in maintaining plasma calcium and phosphorus homeostasis in humans. Vitamin D insufficiency and deficiency is a global health issue. Very few foods naturally contain vitamin D; a major source is oily fish such as salmon. Several studies have analyzed vitamin D content in various fish, however studies concerning canned fish are lacking. In particular, this study was interested in evaluating the vitamin D content in canned sardines in not only the whole fish but also in the olive oil or water it was canned in. It was hypothesized that the vitamin D content in sardines canned in water would be greater than sardines canned in olive oil due to the fat-soluble nature of vitamin D to be more easily extracted into olive oil than water. Sardines (~100g) canned in olive oil had a slightly greater vitamin D content than the sardines in water (2,555.6±234.2 and 1,993.7±2,411.3 IUs ($p<0.05$) respectively). An evaluation of the vitamin D_3 content in the olive oil and water used to can the sardines revealed 701.4±471.1 and 149.1±42.2 IUs in the total olive oil and water respectively recovered from the cans. It was determined that of the total vitamin D content in the can (sardines in olive oil or water) 20.9%±12.8% of vitamin D_3 is found in the olive oil compared to only 14.2%±10.4% ($p<0.05$) vitamin D_3 found in water. These

results support the concept that sardines packed in olive oil may have less vitamin D_3 than similar sardines packed in water.

The analysis of the sardines revealed that they had more than 13 times the amount of vitamin D_3 than that is reported in the USDA table of nutritional facts for canned sardines. This could be because the sardines were caught in the summer months when they are more likely to be generating vitamin D_3 as a result of adequate sun exposure. An alternative explanation for this increase in vitamin D_3 content is the process of canning the sardines. Vital Choice, the supplier of the sardines, immediately ices the fish upon retrieval from the ocean (to ensure freshness) and then are canned in less than 5 hours after being caught. This process of freshness preservation could explain why the vitamin D content was so high; possibly an accurate representation of the original vitamin D content in the sardines.

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$1,25(OH)_2D_3$……1,25-Dihydroxyvitamin D_3

7-DHC……7-Dehydrocholesterol

$24,25(OH)_2D_3$……24,25-Dihydroxyvitamin D_3

$25(OH)D_3$……25-hydroxyvitamin D_3

APC……Antigen presenting cell

BU……Boston University

CDC……Centers for Disease Control

DM……Diabetes mellitus

HCL……Hydrochloric acid

HPLC……High performance liquid chromatography

IOM……Institute of Medicine

IU……International Units

PMT……Photomultiplier tube

PTH……Parathyroid hormone

RANK……the receptor activator of nuclear factor kappa-B

RANKL……the receptor activator of nuclear factor kappa-B ligand

USDA……United States Department of Agriculture

UV ……Ultra violet

VDR……Vitamin D receptor

WARF……Wisconsin Alumni Research Foundation

INTRODUCTION

History

Our understanding and discovery of vitamin D evolved from the clinical presentation of rickets. This fifteenth century skeletal disorder became rampant in the 1700s in Europe as industrialization increased smog coverings and tall buildings prevented sunlight from reaching the city.[1,2] In 1822, Sniadecki noticed that children who lived in inner city Warsaw had a higher incidence of rickets. This allowed him to make the observation correlating city dwellers with rickets.[2] Two biochemists, Elmer McCollum from the United States and Sir Edward Mellanby of the United Kingdom, were interested in testing food products as a cure for rickets, in particular, the benefit of cod liver oil consumption. The use of cod liver oil for medicinal purposes dates back to 1782 where Dr. Robert Darby of Manchester, England claimed to have used over 60 gallons of cod liver oil in his hospital each year.[3] It wasn't until 1824 when Dr. Scheutte of Germany documented the use of cod liver oil as a remedy for rickets, rheumatism, and gout.[3] The cod liver oil was also used to treat xerophthalmia, a disorder causing dryness and irritation of the eyes. However, the benefits of cod liver oil would have been forgotten if it weren't for McCollum and Mellanby who resurrected its use a century later. Mellanby tested the benefit of cod liver oil consumption in dogs kept indoors on a diet of oatmeal, in an attempt to cure rickets[4]. They noted the calcium-deficient dogs presented with deformities in the epiphyses of their long bones; however when these dogs consumed cod liver oil these deformities began to disappear.[5] Thus they concluded that either vitamin A or

one or more other substances in the cod liver oil assisted in the homeostasis of plasma calcium concentrations. McCollum then began his analysis on cod liver oil and various fats and oils to determine what substance was initiating the calcium deposition in bones. He oxidized the cod liver oil for ten hours, which he knew would destroy the vitamin A as a result of its inability to resolve xerophthalmia, and noticed that the cod liver oil was still able to induce calcium deposition in bones. This result made him aware that a substance besides vitamin A was responsible for the calcium deposition, which he called vitamin D.

Meanwhile, an entirely different cure for rickets was observed, the role of ultraviolet (UV) radiation. In 1919, Huldshinsky noted that children who were exposed to a mercury arc lamp showed significant radiologic improvements of their rickets several months later. He noted that exposure to one forearm of a child with rickets displayed the same radiologic improvements in the other arm not exposed to the mercury arc lamp.[2] He concluded that something was made in the skin that entered the circulation caused widespread improvements in children with rickets. Dr. Alfred Hess, an American physician, decided to investigate the effects of UV radiation on rachitic animals. He fed rats a normal diet, and varied their sun exposure; those rats that were not exposed to the sun, developed rickets.[6] Hess took it one step further and began testing on babies who presented with clinical rickets. These babies were exposed to the sun from one half hour to several hours, and in every instance where heliotherapy was applied, rachitic signs and symptoms diminished.[6]

Kramer and Howland monitored the blood levels of children diagnosed with rickets and noticed a low inorganic phosphate level.[7] They treated these children with cod liver oil, just like Scheutte, and they observed that the inorganic phosphate levels began to rise back to a normal level of 4 mg/dL. Just like Kramer and Howland, Hess also saw a rise in inorganic phosphate in children who received heliotherapy for treatment of rickets.[6] Thus there appeared to be a connection between cod liver oil treatment and heliotherapy in curing rickets.

Once Hess made his discovery about metabolic changes as a result of heliotherapy, he brought into question the chemical structure of the antirachitic product formed as a result of irradiation of a substance thought to be cholesterol[8]. He consulted with Adolf Windhaus, a German chemist, to collaborate on discovering the chemical structure of the product in question. Hess and Windhaus discovered

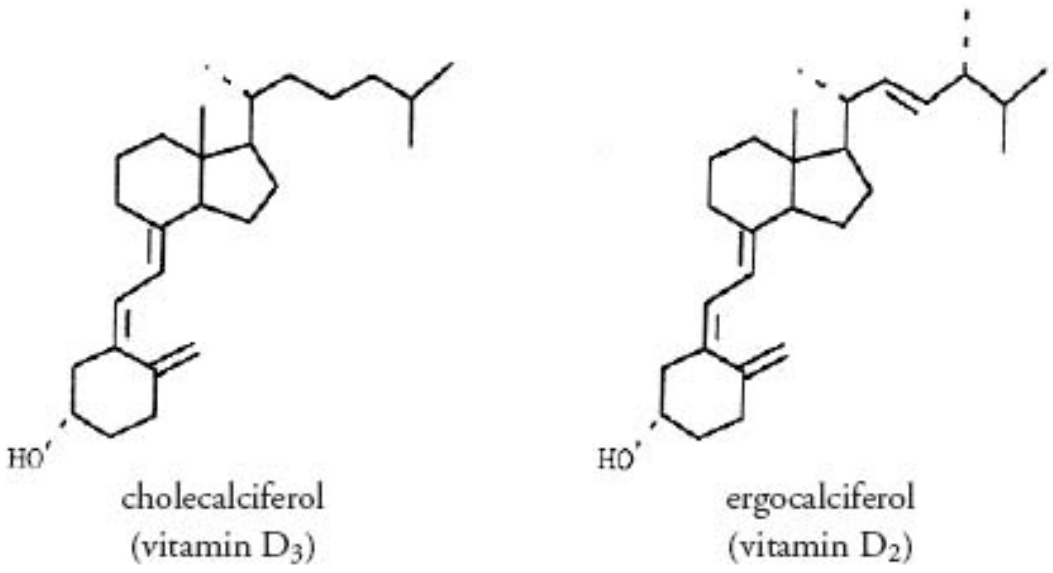

Figure 1: Structures of vitamin D_3 and vitamin D_2 Copyright McLaughlin and Holick (Food and Agriculture Organization)

that cholesterol was not in fact the substance generating antirachitic properties but rather its precursor.[8] At the same time, Rosenheim, a scientist from London, discovered that an impurity of cholesterol could be converted to an antirachitic substance in the presence of UV light. [8] This discovery led to the discovery of the vitamin D precursor or provitamin D. Windhaus continued to study and evaluate this provitamin and eventually won the Nobel Prize in 1928 for determining the structure of 7-dehydrocholesterol (7-DHC) and vitamin D.[8] The vitamin D that Windhaus discovered is known today as vitamin D_3. There are several forms of vitamin D; vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol). Vitamin D_3 is generated from the precursor 7-DHC in the skin following exposure to UVB radiation. Vitamin D_3 is also ingested from certain foods, including fish and cod liver oil. Vitamin D_2 is derived from the UV irradiation of ergosterol found in yeast. [9] This UV exposure to fortified foods with ergosterol and 7-DHC was patented by biochemist Harry Steenbock from the University of Wisconsin. Since the university was not interested in Steenbock's patent, he initiated a non-profit organization, the Wisconsin Alumni Research Foundation (WARF) which funds research from the University of Wisconsin to help commercialize technology generated from research.[10] The difference between vitamin D_3 and vitamin D_2 is that the side chain for vitamin D_2 has a double bond between C-22 and C-23 and a methyl group on C-24 (Figure 1).

Vitamin D production in early life forms

The origin of vitamin D can be traced back millions of years. Phytoplankton and

zooplankton have been producing vitamin D for more than 500 million years.[11] Holick et al reported that a particular phytoplankton that has existed unchanged in the Atlantic ocean for more than 500 million years when exposed to UV radiation produced previtamin D_2 (a precursor of vitamin D_2).[11] Rao and Raghuramulu, reported vitamin D concentrations in zooplankton and phytoplankton. They found that zooplankton and phytoplankton contained 7176 ng/g and 3889 ng/g of vitamin D_2 respectively and 46,238 ng/g and 23,581 ng/g of vitamin D_3 respectively.[12] To put this in perspective, on average, an everyday 1000 IU vitamin D supplement contains 25,000 ng of vitamin D_3. They hypothesized that because zooplankton consume phytoplankton as one of their dietary requirements, they indirectly have a higher concentration of vitamin D.

Vitamin D Metabolism

Both vitamin D_2 and vitamin D_3 are metabolized in the same manner in regards to vitamin D activation. When ultraviolet B (UVB) radiation (wavelength of 290-315 nm) is absorbed into the skin, it converts 7-DHC, which is predominantly located in the stratum spinosum and stratum basale[13], to previtamin D_3, which is thermodynamically unstable. Over several hours it isomerizes into vitamin D_3.[14] The vitamin D_3 from the skin and the vitamin D_2 from food and supplements enters the circulation and are hydroxylated in the liver by a 25-hydroxylase to become 25-hydroxyvitamin D (25(OH)D), which is the major circulating form of vitamin D.[15,14] 25(OH)D is hydroxylated again in the kidneys by 1alpha-hydroxylase to become 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), which is the active form of vitamin D

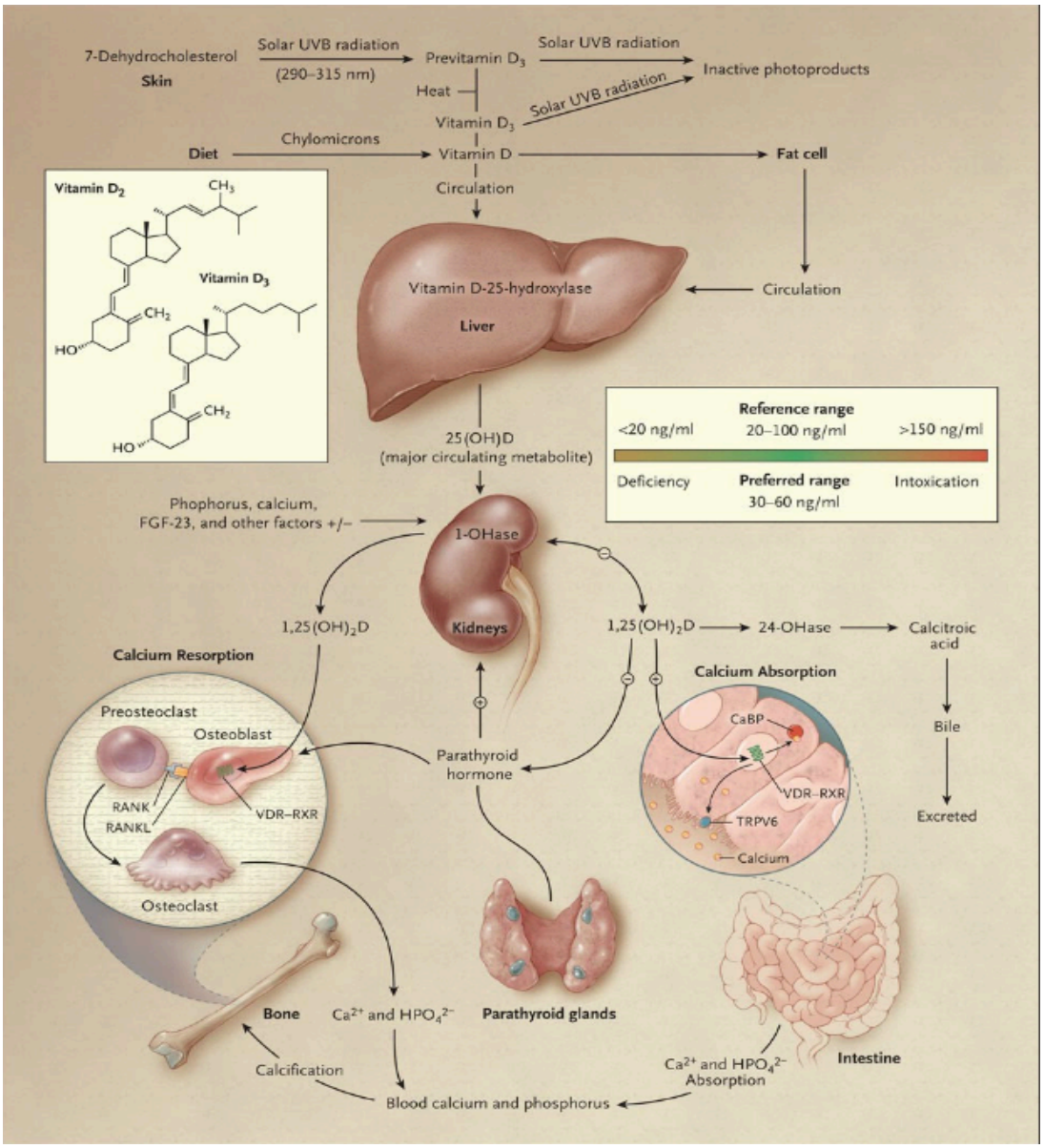

Figure 2: During exposure to solar ultraviolet B (UVB) radiation, 7-dehydrocholesterol in the skin is converted to previtamin D3, which thermodynamically stabilizes to vitamin D_3. Any vitamin D_3 ingested is packaged in chylomicrons and joins with endogenous vitamin D_3 at the hepatic circulation. It is then hydroxylated via vitamin D-25-hydroxylase to make 25(OH)D, the major circulating metabolite, which is used to monitor vitamin D levels in the blood. 25(OH)D is converted to 1,25 dihydroxyvitamin D ($1,25(OH)_2D$) vita 1-alpha hydroxylase in the kidney, the active form of vitamin D. $1,25(OH)_2D$ increases blood calcium and phosphate levels by increasing absorption out of the gut and kidneys. In excess it will also activate RANKL, a ligand on osteoblasts, which will activate RANK on preosteoclasts to mature the cell into active osteoclasts, inducing calcium reabsorption out of bones. (This figure was taken from Holick 2007 "Vitamin D Deficiency")

(Figure 2). When calcium levels are too high, 24-hydroxylase activity increases, hydroxylating C24 and making, 24,25-Dihydroxyvitamin D ($24,25(OH)_2D$) and 1,24,25-trihydroxyvitamin D ($1,24,25\text{-}(OH)_3D$). This step begins the process of the degradation of both metabolites into inactive water-soluble metabolites that can be excreted in the bile.

Vitamin D_2 and vitamin D_3 in the human body. There is much controversy as to which form of vitamin D works best in the body. A study looked at a population of adults, 60% of whom were vitamin D deficient, defined as having a 25-hydroxyvitamin D_3 ($25(OH)D_3$) level <20ng/mL, placed into three cohorts: administered 1000 IUs of Vitamin D2, 1000 IUs of vitamin D_3, or 500 IUs of both. The subjects' levels of $25(OH)\ D_3$ (most abundant circulating form of vitamin D) were assessed to see which form of vitamin D was more influential in the body. They found that ingesting either vitamin D_2 or vitamin D_3 had equal effects on maintaining serum $25(OH)\ D_3$ levels (Figure 3). The study concluded that both vitamin D_2 and vitamin D_3 (Figure 3) were just as effective in maintaining 25(OH)D status [9].

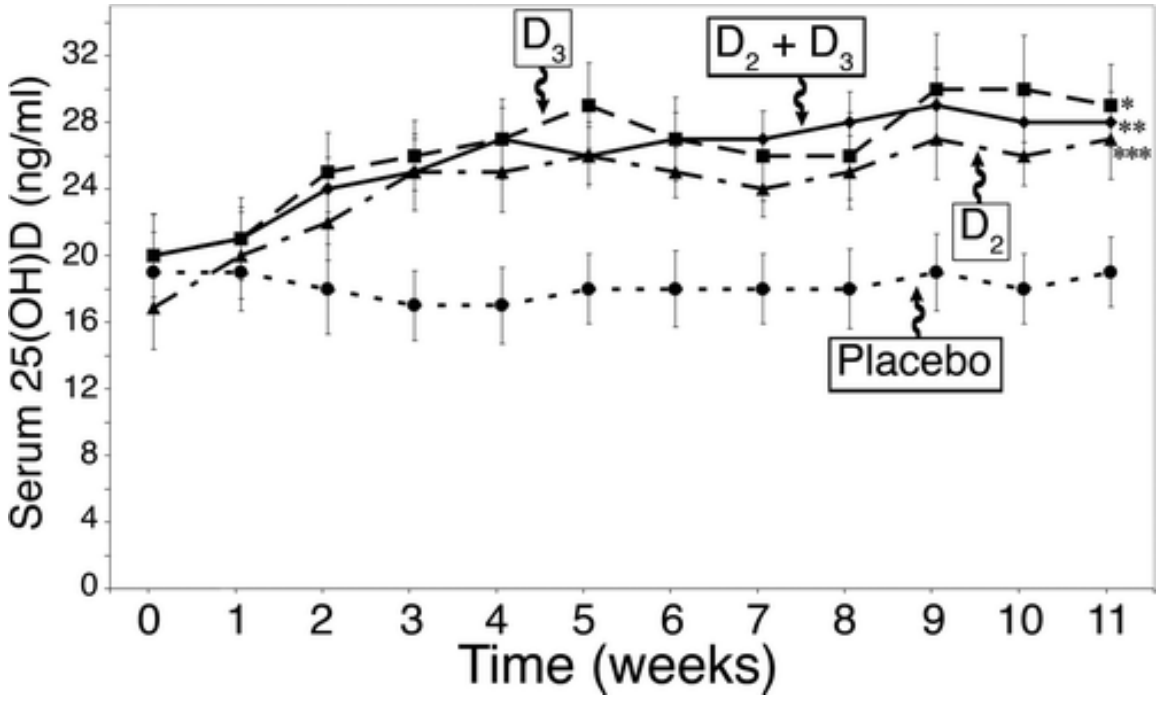

Figure 3: Level of serum 25(OH)D in response to ingesting 1,000 IUs of vitamin D_2 or vitamin D_3 (Holick et al.)

Calcemic Functions of Vitamin D

Vitamin D is a multifaceted hormone, which not only maintains sufficient calcium and phosphate levels in the blood, but also acts on various tissues in the body. Depletion of this hormone can lead to many negative consequences and without the proper supplementation, could be detrimental to health. The activated form of vitamin D, $1,25(OH)_2D$, acts on its vitamin D receptor (VDR) which is located in various tissues and cells in the body, namely, osteoblasts, brain, skin, breast, prostate, colon, and activated B and T lymphocytes [15].

The main function of vitamin D is maintaining blood calcium and phosphorus balance and bone health. Vitamin D has three main targets, the intestine, the kidneys, and the bones, which help regulate calcium and phosphorus in the body.

Calcium is the fifth most abundant element in the human body and is a key player in maintaining physiological functions. In order to perform these functions, calcium needs to be ingested from the diet. As the body's demand for calcium increases the synthesis of 1,25(OH)D rises to increase the efficiency of calcium absorption.[16] Vitamin D is needed for three modes of transport of calcium across the intestinal border: 1) the entry of calcium from the intestinal lumen into the apical membrane of the intestinal cell, 2) intracellular diffusion of the calcium through the cell, and 3) transport of the calcium out of the cell and into the blood. Most of the calcium absorption occurs in the proximal intestine (duodenum), but studies have shown that there is absorption along the entirety of the small intestine. $1,25(OH)_2D$ can also enhance dietary phosphorus absorption in the small intestine by enhancing sodium-dependent phosphorus influx into the intestinal cells[16].

Renal reabsorption of calcium plays a key role in calcium homeostasis. 98% of ionized calcium that enters the glomerulus is reabsorbed[15]. Most calcium reabsorption occurs in the proximal tubule (65%) and occurs via passive diffusion[16]. The rest occurs in the distal tubule where $1,25(OH)_2D$ acts to regulate calcium reabsorption. Sufficient levels of $1,25(OH)_2D$ increases the dietary calcium absorption in the intestines and reabsorption in the kidneys to maintain plasma calcium homeostasis and prevents the need of removing calcium from its storage pools in the bone.[17]

In vitamin D deficiency there is a decrease in blood calcium resulting in the production and release of parathyroid hormone (PTH), stimulating the kidneys to

produce more $1,25(OH)_2D$ (Figure 2). Circulating $1,25(OH)_2D$ binds to the VDR located on osteoblasts which activates the nuclear factor kappa-B ligand (RANKL). RANKL is the ligand for the receptor activator of nuclear factor kappa-B (RANK), located on preosteoclasts. When RANK binds to RANKL it promotes osteoclast differentiation and an increase in bone osteoclastic (bone resorption) activity[18]. Activated osteoclasts releases hydrochloric acid (HCl), dissociating the calcium from the bone matrix and releasing calcium into the blood. Both $1,25(OH)_2D$ and PTH activate RANKL to promote osteoclast maturation and bone resorption. The small intestine, kidneys, and bones therefore use $1,25(OH)_2D$ to maintain optimal serum calcium levels.

Non-Calcemic Functions of Vitamin D

The presence of VDR in various tissues implies that vitamin D has a direct influence on the growth and function of these tissues. For example, a study looked at the effect of $1,25(OH)_2D$ on the foxO transcription factors that control cell growth and proliferation. FoxO is a tumor suppressor and reduces cell proliferation. The authors found that ligand-bound-VDR enhanced the post-translational modification of foxO proteins, increasing the binding to promoter regions for foxO. In the presence of ligand-activated VDR, the effects of foxO were enhanced. In the absence of activated VDR, foxO expression is reduced and cell growth and proliferation ensues[19]. Another study looked at the role of vitamin D intake and incidence of colon cancer. Two groups of mice, one that was fed a regular diet (1 IU/g of vitamin D), the other was fed a diet with a high vitamin D concentration (5 IU/g of vitamin D). After

inoculation with the bacteria *H. bilis*, the mice were examined for inflammation, dysplasia, and neoplasia. 11% of the mice that were fed the high vitamin D diet presented with colon cancer compared to 41% of the mice on a normal diet[20]. The researchers determined incidence of colon cancer by analyzing p53/MAP kinase (well-known tumor suppressors). They found that mice that were fed a high vitamin D diet had decreased MAP kinase activity in colonic cells, thus reducing cell proliferation[20]. Thus the VDR located on the colon has a role in using $1,25(OH)_2D$ to regulate cell growth and proliferation. Overall, these studies provide evidence that in a mouse model, a high intake of vitamin D reduces cell growth and proliferation and the incidence of neoplasm.

Vitamin D also has an influence on fighting infections. For example, VDR on promyeolcytes help suppress proliferation of promyelocytes and cause their differentiation into monocytes.[21] Monocytes are the precursors to macrophages and dendritic cells whish are antigen-presenting cells (APC) that assist in innate immunity. $1,25(OH)_2D$ interacting with the VDR in macrophages has the ability to induce transcription of 1α-hydroxylase genes to increase 1-alpha hydroxylase converting 25(OH)D to $1,25(OH)_2D$. This $1,25(OH)_2D$ can exit the macrophages and induce activity on T lymphocytes to generate cytokines and on B lymphocytes to regulate production of antibodies. [14] Thus vitamin D also plays a role in assisting with immunity and fighting infection. Another aspect of vitamin D is its role in Type I diabetes mellitus (DM). Association studies have shown that infants who take

vitamin D in their first year of life reduced their risk of developing Type I DM by 88% 31 years later. [22]

Vitamin D can also play a major role in the prevention of cardiovascular disease. Vascular smooth muscle cells and endothelial cells both express 1α-hydroxylase. Thus, vitamin D plays a role in vascular function by inducing growth of both cell types as well as inducing vasodilation and reducing expression of thrombogenic genes.[23] Vitamin D can also have widespread effects on systemic vasculature. Mice that over-expressed 24-hydroxylase (reducing the amount of $1,25(OH)_2D$) had a marked increase in the incidence of atherosclerosis. [24] These studies together show that vitamin D is critical in both maintaining optimal serum calcium and phosphorus levels, as well as regulation of growth, proliferation, and other non-calcemic functions.

Vitamin D Deficiency

Someone who is deficient in vitamin D absorbs approximately 10–15% of calcium and 60% of phosphate from their diet[25]. The interaction of $1,25(OH)_2D$ with the VDR on the intestine increases the efficiency of calcium intestinal absorption to 30–40% and phosphorus intestinal absorption to 80%[25,26]. Vitamin D deficiency can lead to rickets/osteomalacia in two ways: 1) vitamin D deficiency results in a low calcium phosphate product, disabling mineralization of bone matrix, and making the bone less rigid. Those who are vitamin D deficient have an increase PTH as a result of low plasma calcium. PTH induces the maturation of osteoclasts to stimulate the reabsorption of calcium from bone in order to correct the hypocalcemia. However,

PTH also increases renal excretion of phosphorus. Thus, those who are vitamin D deficient have an overactive PTH pathway leading to a decreased plasma phosphorus and reducing the calcium phosphate product; preventing bone mineralization,[15] resulting in rickets/osteomalacia. 2) Vitamin D deficiency increases chondrocyte maturation, which plays a role in calcifying the epiphyseal plates (growth plates).[5,27,28] The calcification of growth plates can lead to stunting or a reduction in vertical growth.

Recommended Daily requirements for Vitamin D

When assessing the optimal dietary intake of vitamin D, there might be some controversy as to which guideline to follow. The Institute of Medicine (IOM) issued guidelines to maintain adequate serum 25(OH)D levels above 20 ng/mL.[29] The Endocrine Society's guidelines reflect the needs to treat and prevent vitamin D deficiency and maintain a 25(OH)D status above 30 ng/mL.[30] Tables A and B present values for required daily vitamin D intake values and the cut-offs for vitamin D status.

Table A: Recommended Daily Intake (IU/day)

Age (years)	Institute of Medicine	Endocrine Society Guidelines
0	600	400–1000
1–18	600	600–1000
18+		1500–2000
70+	800	

Table B: 25(OH)D Levels correlating with status (ng/mL)		
Status	**Institute of Medicine**	**Endocrine Society**
Sufficient	20–50	>30
Insufficient	12–19.6	21–29
Deficient	<12	<20

The Troubling Winter Months

According to the IOM, humans should maintain a 25(OH)D level of at least 20 ng/mL to be vitamin D sufficient.[31] As previously mentioned, in an otherwise healthy individual with adequate sun exposure who consumes food high in vitamin D and takes proper supplementation, 25(OH)D levels are sufficient. The ability to maintain 25(OH)D levels via sun exposure is influenced by the zenith angle of the sun.[32] The zenith angle is defined as the oblique angle between the sun's rays and the earth's surface. The time of day, season, and latitude on the earth's surface all affect the zenith angle. Above 37° latitude during the months of November through February, there is a significant decrease in the amount of UVB photons hitting the earth's surface, as much as 80%–100% depending on latitude [17]. Thus, for those living in areas at a zenith angle incompatible for producing vitamin D in the winter, they need to obtain their vitamin D via supplements and/or food containing or fortified with vitamin D.

Food Sources of Vitamin D

Few foods naturally contain vitamin D (either D_2 or D_3) [11]. Oily fish are a good source of vitamin D. For instance fresh, wild salmon (3.5oz or 100g) has between 600–1000 IU of vitamin D_3. Canned tuna (3.6oz or 102 g) has about 230 IU of vitamin D_3.[11]

There are some non-fish sources of vitamin D such as sun-dried mushrooms and egg yolks. Sun-dried shiitake mushrooms have about 1600 IU of vitamin D_2. Egg yolks have about 20 IU of vitamin D_2 or vitamin D_3 [11]. There are some fortified foods that also contain vitamin D; fortified milk and fortified orange juice each has about 100 IU/8 oz of vitamin D_3. Some cereals, such as Quaker Oatmeal have as much as 40 IU vitamin D/cup.[33] Margarine has about 60 IU of vitamin D/tablespoon.[33] In a study that looked at the plasma levels of 25(OH)D_3 amongst fish eaters and non-fish eaters (Vegans and Vegetarians), those who did not consume fish had lower levels of 25(OH)D_3 in both the summer months (20%) and winter months (38%) [34](Figure 4). Thus the consumption of fish in the diet is critical in maintaining vitamin D status throughout the year, especially during the winter months.

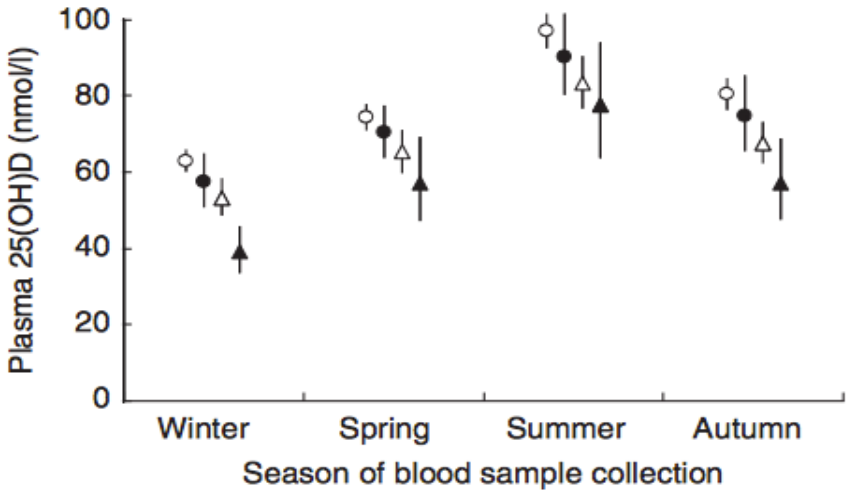

Figure 4: Mean plasma concentrations of 25-hydroxyvitamin D (25(OH)D) in meat eaters (n 1388), fish eaters (n 210), vegetarians (n 420) and vegans (n 89) by season of blood sample collection. Data are presented as geometric mean and 95 % CI adjusted for year of blood sample collection, age, sex, case–control status, BMI, smoking status, summer outdoor activity, vigorous exercise, current use of hormones, supplement use and the interaction of sex and age according to diet group (○, meat eaters; ●, fish eaters; △, vegetarians; ▲, vegans) . Figure obtained from (Crowe et al., 2011).

Source of vitamin D from fish

The Japanese diet contains a large variety of fresh fish and would therefore expect to see adequate plasma 25(OH)D levels in individuals on the Japanese diet. One study looked at the Japanese diet in elderly individuals and correlated it with their plasma 25(OH)D levels. Approximately 31 kinds of fish were consumed during this study and the most common types of fish consumed are chum salmon (1280 IUs vitamin D/100g of fish), baby sardines (1,840 IUs/100 g of fish), mackerel (440 IUs/100 g of fish), and cod (40 IUs/100 g of fish) [35]. This group of researchers looked at 30 residents in a nursing home whose diets were carefully designed and monitored, where each food source was weighed and recorded. The researchers looked at the fish, meat, eggs, and mushrooms consumed to analyze the individual contribution to the total serum 25(OH)D concentration. They found that elderly who consumed fish in their diets obtained 90.7% of their plasma 25(OH)D content from fish, followed by mushrooms (4.4%), eggs (3.2%), and meat (1.7%). Thus they were able to conclude the significant benefit of consuming fish by demonstrating it to be the greatest contributor to total serum 25(OH)D concentrations.

Fish is widely recommended by physicians for its nutritive properties. They contain proteins rich in amino acids, essential elements such as calcium, phosphorus, fluorine, and iodine. Oily fish have fats with essential fatty acids, and of course they have an abundance of vitamins (A and D) [36].

Canned Fish

Unfortunately, many people do not have access to fresh fish like those on a Japanese diet would, and therefore have to rely on canned fish to obtain its nutritive properties, such as being a good source of vitamin D. Canned seafood, one of the oldest industries in America, appeared on shelves in the early 19th century. To this day it is still in high demand. In 2009, sales of canned private-label salmon increased 34.6 percent to $24.7 million and canned private-labeled tuna increased to 17.5% to $226 million [37]. The benefit of consuming fish from a can is its preservation in its natural state. Canned fish also allows for quality control and long shelf life packaging, which allow for areas not close to the sea to consume fish. [38]

However, unlike fish that one would buy from a market, canned fish is stored in olive oil, water, natural juices, or a mix of the three; there additives add another variable to the vitamin D content in canned fish. Understanding the natural properties of vitamin D, being a fat-soluble vitamin, one might assume that the concentration of vitamin D in fish would vary based on the solution in which it is canned. Ninety grams of sardines were reported to have 175.09 IU of vitamin D [39]. However, this calculation was based only on whole sardines and did not take into account how much vitamin D was in the solution that the sardines were canned in. Since vitamin D is fat-soluble, some of the vitamin may be leeched from the fish into the oily suspension in which the fish was canned. Since the suspension is discarded prior to ingestion of the fish, the consumer might not be obtaining all of the vitamin D_3 that was originally in the fish before it was canned.

Prevalence of Vitamin D insufficiency

There is much evidence documenting widespread vitamin D insufficiency and deficiency in children and adults. According to the Center of Disease Control (CDC) in 2001–2006, two-thirds of the population is vitamin D sufficient; which according to the IOM range of acceptable values for 25(OH)D is between 20–50 ng/mL. About 24% of the population was found to be at risk for vitamin D inadequacy (12–19.6 ng/mL), and 8% were at risk for vitamin D deficiency (<12 ng/mL) [40]. The prevalence is lower in white non-Hispanic males as well as pregnant or lactating females.

In a similar study conducted in Spain, 31% and 18% of women were vitamin D insufficient and deficient respectively [41]. They also found that risk of insufficiency and deficiency decreased during the summer months. In Kuwait, 56% and 27% were inadequate and deficient respectively [42]. Interestingly, these individuals were also at an increased risk of type II diabetes, which is associated with vitamin D inadequacy/deficiency. In France, a study looked at those who had 25(OH)D levels below the cut-off for insufficiency (<20ng/mL) and correlated it with dementia. Out of the 288 subjects with vitamin D deficiency, 33% presented with some degree of dementia [43]. In India amongst 5,317 children between the ages of 8–10 years old; the prevalence of vitamin D deficiency (<20 ng/mL) was 10.4% for boys and 11.1% for girls [44]. These studies together show that there is a widespread effect of vitamin D deficiency across the planet and measures need to be taken to reduce this incidence.

Study objective

The objective of this study is to determine the concentration of vitamin D in canned sardines and their respective canned solutions by measuring vitamin D content in the whole fish and the oil or water that the fish was canned in. The hypothesis of this study was that sardines canned in oil would have less vitamin D in the whole fish compared to sardines that were canned in water. The prediction was that more of the vitamin D would be found in the less polar olive oil compared to the water solution that the sardines were canned in. The findings from this study will help provide a greater knowledge regarding the vitamin D nutritive value of canned sardines.

METHODS

Materials

All fish products used in the testing of these experiments were kindly provided by Vital Choice Wild Seafood and Organics located at 2460 Salashan Loop Ferndale, WA 98248. The sardines came in small tins with three sardines per container (~100g). The sardines were obtained from Porto, in the northwest part of Portugal during the summer months. The fish were place on ice immediately following extraction from the ocean. They were then sent to a local Portuguese canning manufacturer where they were canned. The normal phase Zorbax silica 5 micrometer HPLC column used was purchased from Agilent (5301 Stevens Creek Blvd, Santa Clara, CA 95051, United States). The reverse phase C-18 HPLC Vydac column used was purchased from Grace (7500 Grace Drive, Columbia, MD, 21044, United States). The Agilent 1100 Series HPLC machine from Hewett Packard (5301 Stevens Creek Blvd, Santa Clara, CA, 95051, United States).

Vitamin D_3

Vitamin D_3 (100 ng) prepared from Roche vitamin D_3 crystals (430 E 29th St, New York, NY 10016) was used as the control sample and a stock solution of 2mg/L. This was prepared by adding 2mg of vitamin D_3 to a liter of 100% ethanol. Twenty μL of the stock solution (40ng) was placed in a test tube and dried down with nitrogen. After drying, 140μL of normal phase running solution (0.8% isopropanol in hexane) was added to the test tube. 140μL was transferred into a 1.5 mL high performance

liquid chromatography (HPLC) running tube and placed into the HPLC. The automated injector injected the standard vitamin D_3 onto the column and was eluted for 15 minutes at a flow rate of 1.5 mL/min. This sample passed through a variable wavelength detector at 265 nm allowing the detection of compounds that absorbed at this wavelength. The wavelength of 265 nm was chosen because it is the maximum absorption for vitamin D. The HPLC displayed: 1) A chromatogram of all compounds that absorbed at 265 nm and the times at which the compounds eluted; the greater the amplitude the greater concentration of vitamin D. 2) A UV absorption spectrum for selected peaks of interest from the chromatogram. Vitamin D_3 has a unique absorption spectrum that peaks at 265 nm and has a trough at 228 nm. Knowing the unique absorption spectrum helped identify the peak in the chromatogram as vitamin D by scanning each peak for characteristics resembling the known absorption spectrum for vitamin D. This matching process allowed the determination of the time at which vitamin D_3 in the samples eluted from the HPLC column. This time was used as a standard and applied to the experimental samples to determine when to collect the eluting vitamin D_3 for further analysis.

The Use of Radioactive vitamin D_3

To accurately determine the recovery of vitamin D, 10 μL of ^{3}H-vitamin D_3 (8000 cpms; counts per minute) were added to each saponification tube as well as to three separate counting vessels for recovery purposes. The use of adding radioactive vitamin D_3 to three counting vessels was to determine the cpms of ^{3}H-vitamin D_3 that would be added to the samples and compare thus number to how many cpms

were recovered at the end of the experiment. Thus, the use of radioactive ^{3}H-vitamin D_3 provided the ability to determine the percent recovery of the vitamin D_3 in the sample at the end of the analysis. This could be used to determine the total amount of vitamin D_3 in the fish sample.

Saponification and lipid extraction of canned sardines

All of the fish (On average 3/can) were removed from the can and placed in a small dish where they were cleaned with 10 mL of ethanol to remove the olive oil from the fish. The ethanol was discarded and the fish were weighed and placed into a mortar and pestle where it was mashed for 10 minutes into a paste at room temperature. One-gram samples of the fish paste were placed into a 50 mL sealable glass test tube to prepare for saponification. Saponification was performed by dissolving three grams of potassium hydroxide (KOH) in 5mL of water. This was added to the 50 mL sealable glass tube and vortexed for 30 seconds. Seven mL of 100% ethanol were added to the 50 mL tubes, and the resulting mixture was vortexed for another minute to homogenize the mixture. Ten µL of ^{3}H-vitamin D_3 of 8000 cpms was added to the mixture and the solution was vortexed for 30 seconds. The tube was sealed using its fitted cap and wrapped in parafilm. The tubes were placed on a shaker for eight hours at room temperature on medium speed. Once that time elapsed, 15 mL of hexane was added to the mixture, and shaken for 30 minutes. The tubes were left to settle for 2 minutes for phase separation and then the hexane layer was removed and placed into a separatory funnel. Another 15 mL of hexane was added to the original 50 mL tube, shaken for 30 minutes, settled for 2 minutes

for phase separation, followed by the transfer of the hexane layer to the separatory funnel. After the second transfer, the separatory funnel contained approximately 30 mL of hexane. 100 mL of distilled H_2O was added to the separatory funnel, sealed

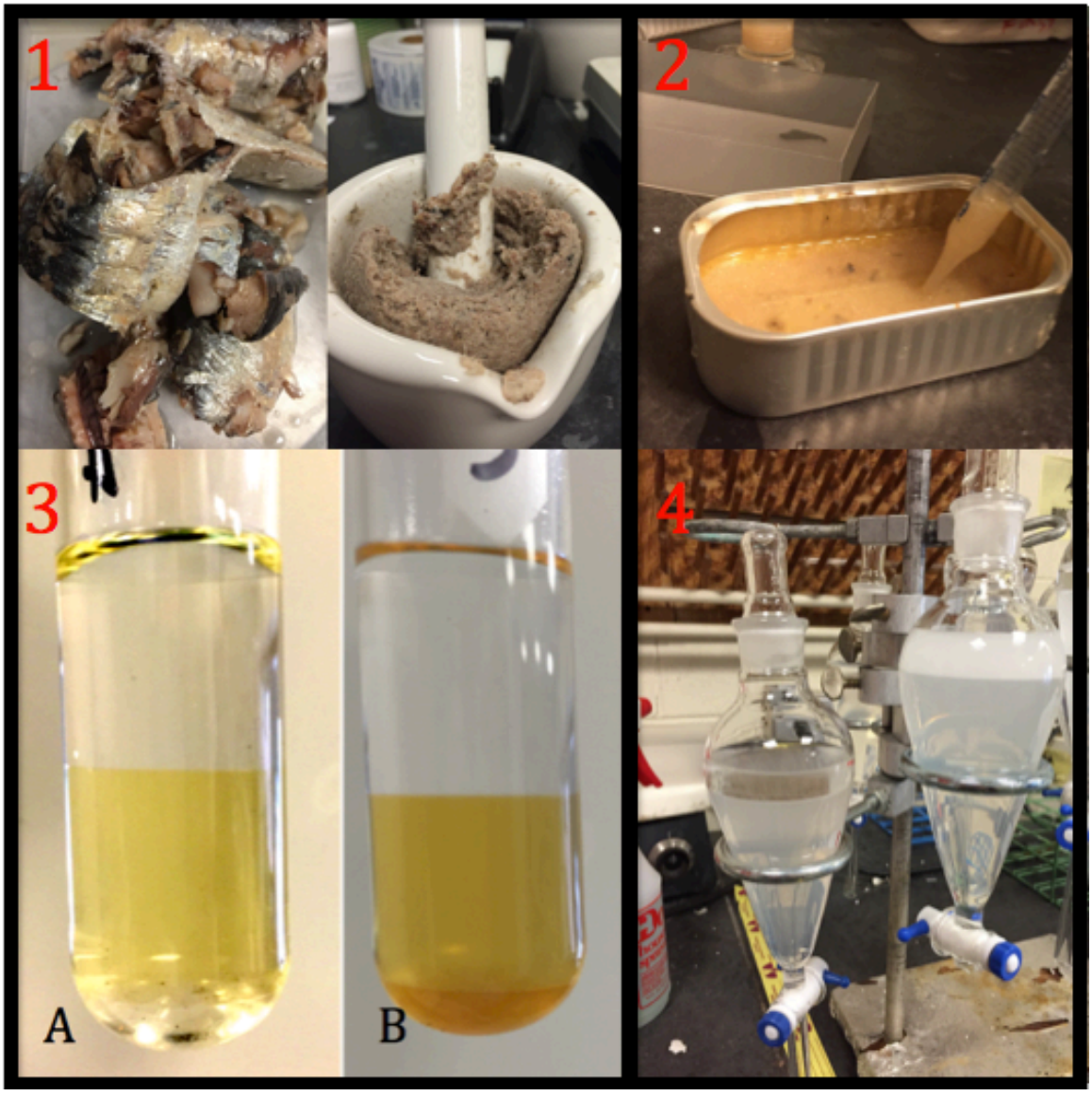

Figure 5: 1) A sardine on the left directly from can; sardines on the right after mashing into paste like consistency. 2) Extraction of oil solution after removing fish meat from container. 3) Separation of layers after hexane was added and solution was shaken for 30 minutes. Tube A contains the oil suspension, Tube B contains the fish meat (note the settled fish particles at the bottom of tube B). The nonpolar solution containing the vitamin D is the top layer. 4) Hexane layers added to separatory flasks. Note the top layer containing hexane. The bottoms layer, polar water solution, was then washed out by opening the blue valve.

with a glass cap and parafilm, and placed in the shaker for 30 minutes. The separatory funnel was then oriented vertically and the contents allowed to settle for 1–2 hours. Once the layers had separated and a clear separation of layers was visible, the water portion (lower layer) was removed. This wash procedure was performed three times. The hexane layer, approximately 30 mL, was then removed and placed into 50 mL glass tubes where the solution was dried with nitrogen gas.

Saponification and lipid extraction of canned medium

After the fish were removed, the medium remaining in the can was collected and the volume was measured. The solution was either an oily solution or a watery solution. A 5mL mixed sample was taken and placed into small glass tubes and centrifuged at 4000 rev/min for 5 minutes allowing for any residual fish particles to settle to the bottom. 1 mL samples were collected and placed into 50 mL sealable tubes where the rest of the saponification procedure was carried out in the same way as the fish.

Preparation of Straight Phase HPLC

The samples were dried down under nitrogen and the walls of the tube were washed with 1 mL of running solution (0.8% isopropanol in hexane) and washed again with 0.75 mL of running solution to recover the lipid extract. The solution was pipetted out and placed into a centrifuge tube and centrifuged for five minutes at 4000 rev/min. The solution was removed and placed into a small 10x75 mm glass tube leaving behind the solid particulate materials at the bottom of the centrifuge tube. The solution was dried down with nitrogen. 140 μL of running solution was added to the small tube and pipetted into an HPLC-Agilent 1.5 mL tube. Prior to

running the experimental samples, a sample of the standard vitamin D_3 was run on the HPLC to obtain a baseline reading to determine the time at which the vitamin D_3 eluted. The HPLC was set to run each sample for 18 minutes with a flow rate of 1.5 mL/min. The effluent from the HPLC was collected into counting vessels at the time between 10.5 and 12.5 minutes, the same time when the standard vitamin D_3 eluted. The collected samples were collected into counting vials and dried down under nitrogen. 0.5 mL of 100% ethanol and 10 mL of scintillation cocktail were added to each vial. The vials were sent to a radiation lab along with the three control vials for liquid scintillation counting. The scintillation cocktail solvent absorbs the beta particles of the ^{3}H-Vitamin D_3. This excitation energy is transferred to a scintillation solute which results in a flash of light (scintillation) when the solute molecules return to ground state. The number of scintillations emitted is proportional to the energy of the beta particle. A photomultiplier tube (PMT) is used to detect and amplify the light photons from the sample. The height of the pulse is proportional to the number of photons, which in turn is proportional to the energy of the beta particles. This value gives us the final cpm of our sample, which was used to generate a recovery of our vitamin D solution.

Calculations

The chromatogram for each sample was compared to the chromatogram for the standard vitamin D_3 in order to determine if a peak was present that migrated like the standard vitamin D_3. The UV absorption spectrum for the vitamin D_3 from the chromatographed samples was compared to the standard absorption spectrum for

vitamin D_3. Once confirmed that vitamin D_3 was present, the peak was integrated and multiplied by a factor of 0.4; a factor used to convert the area into nanograms. Once the cpms for the radioactive vitamin D_3 were obtained, it was possible to calculate the percent recovery and use this to determine the amount of vitamin D_3 that was present in the sample.

RESULTS

Standard Vitamin D_3 Chromatogram and UV Spectrum

Four types of samples were tested for vitamin D content: 1) sardines canned in olive oil 2) sardines canned in water 3) olive oil from a can of sardines canned in olive oil 4) water from a can of sardines canned in water. The four separate samples were evaluated. They were saponified, extracted, and placed on a HPLC. Results were reported as mean ± standard deviation. A standard sample of vitamin D_3 was run on the HPLC prior to running the experimental samples. This was used to determine the elution time and absorption spectrum to use as a comparison to the experimental samples. The absorption spectrum was used to confirm if vitamin D_3 was present in our samples as it provided a characteristic absorption (peak at 265 nm and trough at 228nm). Additionally the ratio of the peak to trough was a 3/2 ratio. A standard set of vitamin D_3 was performed prior to each separate sample. The chromatograms and UV spectra can be found at the beginning of each section.

Vitamin D_3 content from Sardines canned in Olive oil

Three sardines that were recovered from a can containing olive oil were mashed into three 1-gram samples and saponified, extracted, and run through HPLC to determine their vitamin D_3 content. It was observed that the lipid extract from the saponified sardines showed a vitamin D_3 peak that eluted at 11.90 minutes (Figure 7). This time is similar to the eluted peak for the standard D_3 (Figure 6a). The UV absorption spectrum showed a peak at 265 nm and a trough at 230 nm (Figure 8),

characteristics for vitamin D_3, similar to that of the standard vitamin D_3 sample (Figure 6b).

The area under the peak was converted to micrograms of vitamin D_3 to determine how much vitamin D_3 was in the sample. The tritium that was collected was counted to determine the starting concentration of vitamin D_3. The vitamin D_3 concentrations for sardines canned in olive oil (three cans) can be found in Table 1A. The average vitamin D_3 content can be found in Table 1B. The average concentration of vitamin D_3 in a 1 gram sample from the whole sardine and the average concentrations of vitamin D_3 in 1 can of sardines in olive oil are shown in Tables 1B and 1C respectively.

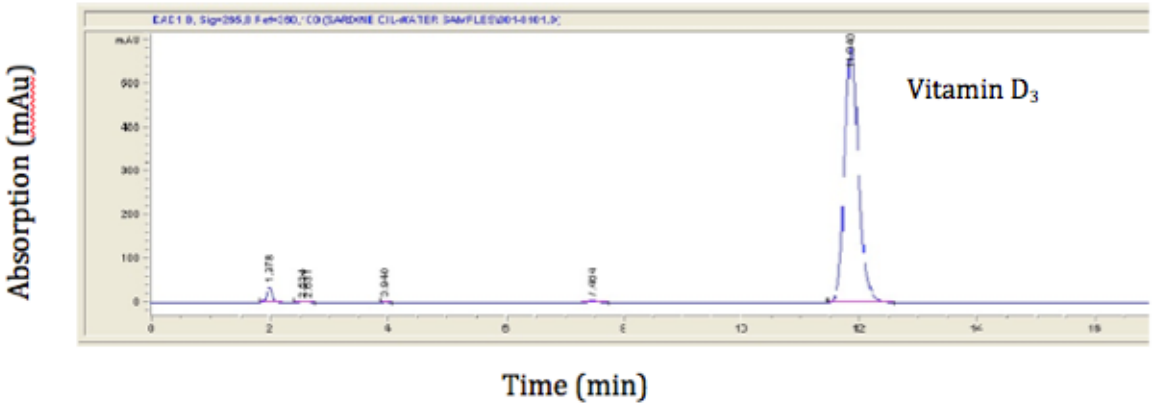

Figure 6a: Control chromatogram of standard vitamin D_3 in 0.8% IPA in hexane on straight phase HPLC detected at 265 nm (prior to running samples of sardines canned in olive oil).

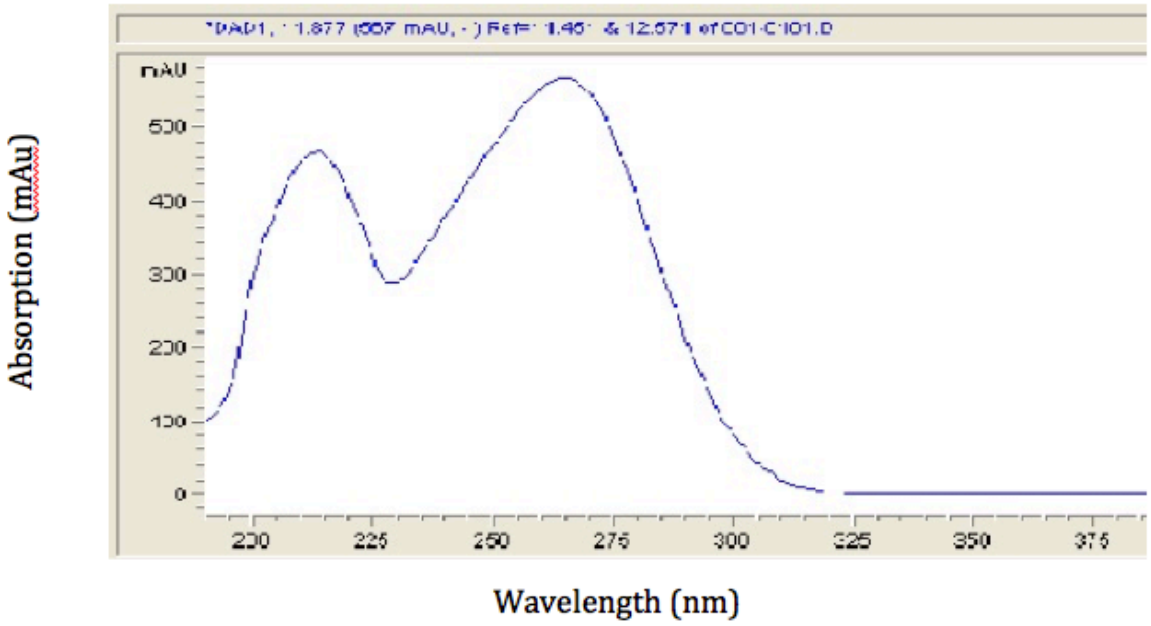

Figure 6b: UV absorption spectrum recorded from the peak that eluted at 11.89 min from figure 6a

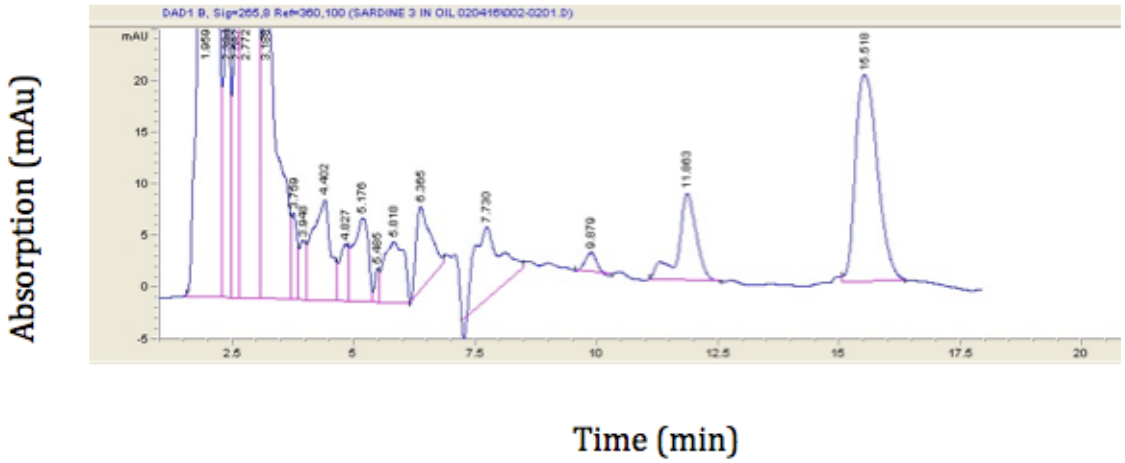

Figure 7: Lipid extract from a whole sardine that was canned in olive oil that was chromatographed on straight phase HPLC with 140 µL of 0.8% isopropanol in hexane at a flow rate of 1.5 mL/min

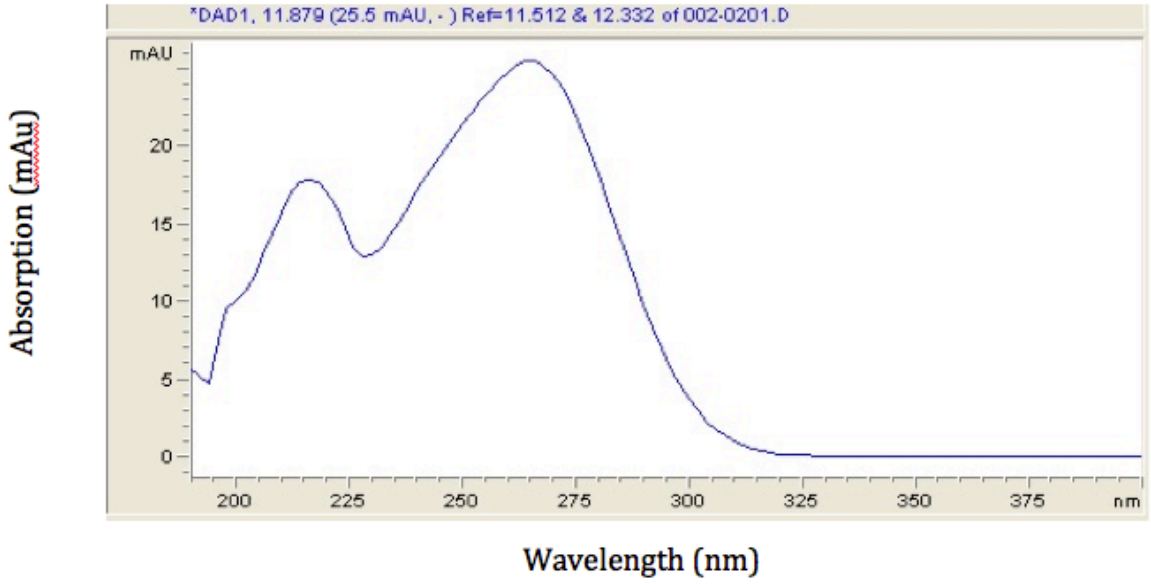

Figure 8: UV absorption spectrum recorded from the peak that eluted at 11.9 min from figure 7

Table 1A: Vitamin D_3 concentration in 1 g samples of canned sardine in olive oil				
	Weight of sardine sample (g)	% Recovery	Vitamin D_3 (μg/g)	Vitamin D_3 (IU/g)
Can 1	1.01	37.4	0.55	21.92
	1.00	35.7	0.27	10.85
	1.00	33.6	1.29	51.49
	Mean±SD		**0.70±0.53**	**28.1±21.0**
Can 2	1.00	11.7	0.77	30.7
	1.01	15.3	0.67	27.0
	1.00	17.4	0.50	20.2
	Mean±SD		**0.65±0.13**	**26.0±5.3**
Can 3	1.00	36.2	0.69	27.5
	1.00	9.4	0.55	21.8
	1.00	21.0	0.68	27.3
	Mean±SD		**0.64±0.08**	**25.5±3.2**

Table 1B: Vitamin D_3 content in 1 g samples from three cans of whole sardines in olive oil		
Can	Vitamin D_3 (μg/g)	Vitamin D_3 (IU/g)
1	0.70	28.1
2	0.65	26.0
3	0.64	25.5
Mean±SD	**0.66±0.35**	**26.5±1.4**

Table 1C: Total vitamin D_3 content/can of sardines canned in olive oil			
Can	Mass of total sardines/can (g)	Vitamin D_3 (μg/can)	Vitamin D_3 (IU/can)
1	94.5	66.36	2,655.5
2	88.0	57.09	2,288.0
3	106.8	68.11	2,723.4
Mean±SD	**96.4**	**63.85±5.92**	**2,555.6±234.2**

From Tables 1A–C an average 1g sample of sardine submersed in oil had an average of 0.66±0.035 μg of vitamin D_3 (25.5±1.4 IU). The sardines in each can were weighed, with an average weight of 96.4 g of sardine/can. In each can, an average of 63.85±5.92 μg of vitamin D_3 (2,555±234.2 IU) was present.

Vitamin D_3 content in sardines canned in Water

Three sardines that were recovered from a can of sardines canned in water were mashed into three 1-gram samples and saponified, extracted, and run through HPLC to determine their vitamin D_3 content. It was found that the vitamin D_3 peak eluted at 10.9 minutes (Figure 10). This time is similar to the eluted peak for the standard D_3 (Figures 9a). The UV absorption spectrum showed a peak at 265 nm and a trough at 230 nm (Figure 11), characteristics for vitamin D_3 found in the standard sample (Figure 9b). The water extracts were run in triplicate and the chromatograms and

UV absorption spectra for each sample were very similar with troughs and peaks at the correct wavelength.

The area under the peak was converted to micrograms of vitamin D_3 to determine how much vitamin D_3 was in the sample. The tritium was collected and counted to determine the starting concentration of vitamin D_3. The vitamin D_3 concentrations (three cans) can be found in Table 2A. The results in Table 2B show the average concentration of vitamin D_3 in a 1-gram sample of whole sardine in water and Table 2C displays average concentrations of vitamin D_3 in 1 can of whole sardine in water.

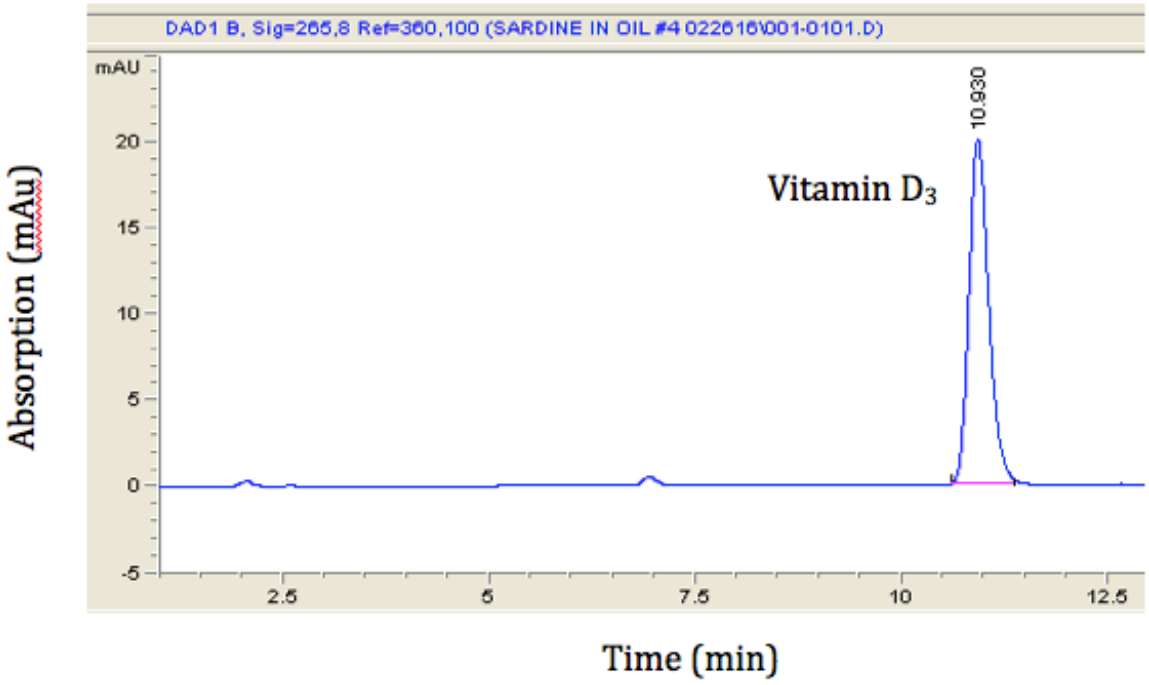

Figure 9a: Control chromatogram of standard vitamin D_3 in 0.8% IPA in hexane on straight phase HPLC detected at 265 nm (prior to running samples of sardines canned in water).

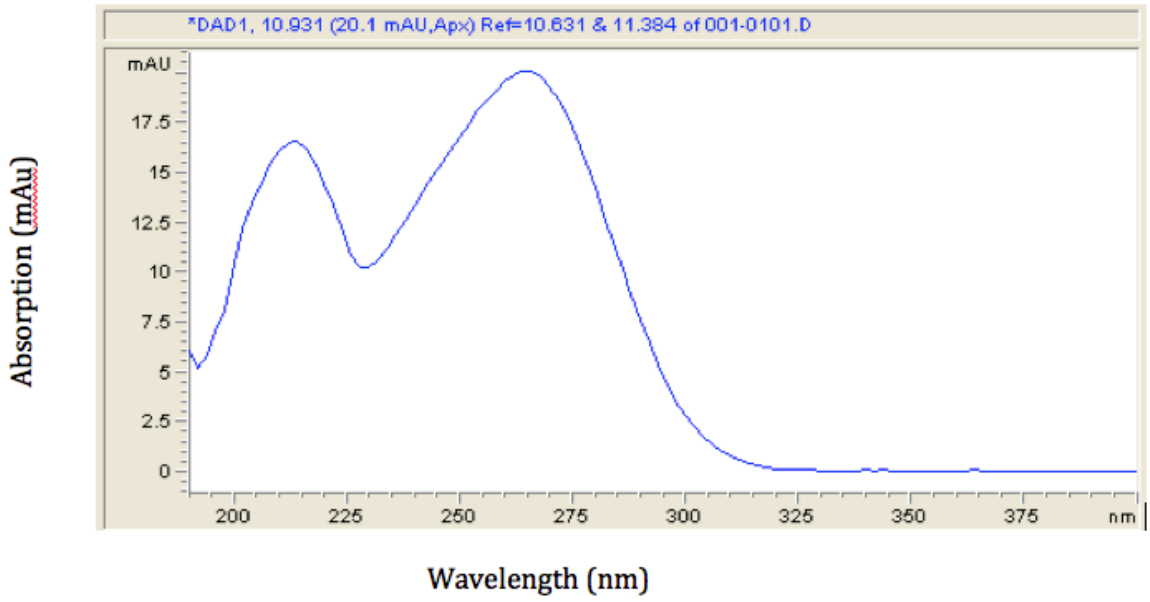

Figure 9b: UV absorption spectrum recorded from the peak that eluted at 10.9 min from figure 9a

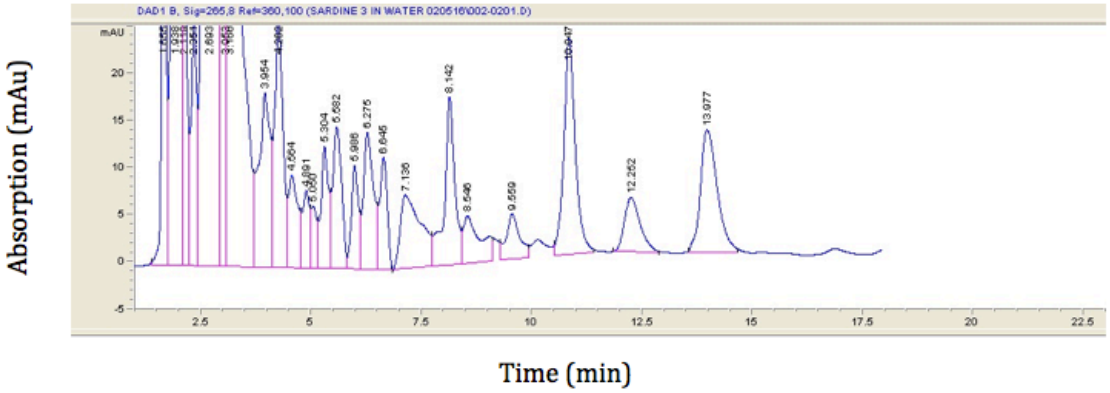

Figure 10: The lipid extract from sardines that were canned in water and were chromatographed on straight phase HPLC with 140 µL of 0.8% isopropanol in hexane at a flow rate of 1.5 mL/min

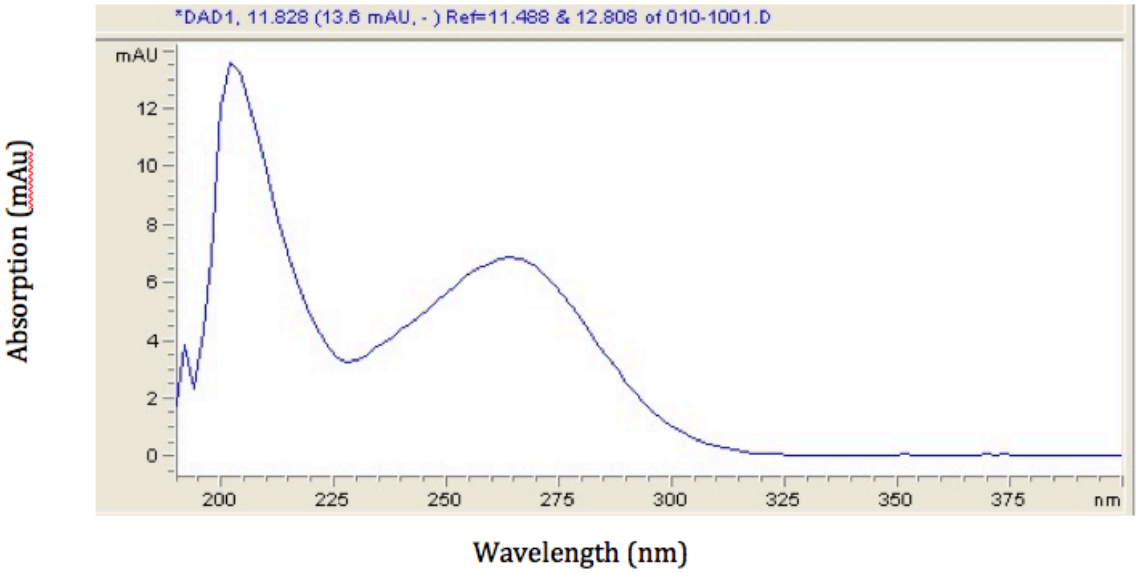

Figure 11: UV absorption spectrum recorded from the peak that eluted at 10.9 min from figure 10

Table 2A: Vitamin D_3 concentrations of 1 g samples of sardines canned in water				
	Weight of sardine sample (g)	% Recovery	Vitamin D_3 (µg/g)	Vitamin D_3 (IU/g)
Can 1	1.01	37.8	0.16	6.4
	1.00	48.1	0.17	6.7
	1.00	46.8	0.13	5.4
	Mean±SD		**0.15±0.02**	**6.2±0.7**
Can 2	1.00	12.2	1.37	54.6
	1.01	14.5	1.42	56.8
	1.00	21.0	1.05	41.8
	Mean±SD		**1.28±0.20**	**51.1±8.1**
Can 3	1.00	39.5	0.16	6.3
	1.01	27.4	0.15	6.1
	1.00	36.4	0.15	6.2
	Mean±SD		**0.15±0.001**	**6.2±0.1**

Table 2B: Vitamin D_3 content in 1 g samples from three cans of whole sardines canned in water		
Can	Vitamin D_3 (μg/g)	Vitamin D_3 (IU/g)
1	0.15	6.2
2	1.28	51.1
3	0.15	6.2
Mean±SD	**0.53±0.65**	**21.2±25.9**

Table 2C: Total vitamin D_3 content/can of sardines canned in water			
Can	Mass of total sardines/can (g)	Vitamin D_3 (μg/can)	Vitamin D_3 (IU/can)
1	91.5	14.08	567.3
2	93.5	119.44	4,777.85
3	102.6	15.84	636.1
Average	**95.9**	**49.79±60.33**	**1,993.7±2,411.3**

From Tables 2A–C the average 1g sample of sardine meat submersed in water had an average of 0.53±0.65 μg of vitamin D_3 (21.2±25.9 IU). The sardines from each can were extracted and weighed, with an average weight of 95.9 g of sardines/can. In each can, an average of 47.79±60.35 μg of vitamin D_3 (1,993±2,411.3 IU) was present. The standard deviations are large for this group because in can 2 (Table 2A) the amount of vitamin D that was determined was much higher than what was found in cans 1 and 3.

Vitamin D_3 content in olive oil from canned sardines in olive oil

Olive oil extracted from a can of sardines canned in olive oil was saponified, extracted, and run through HPLC to determine its vitamin D_3 content. It was found that the olive oil from the canned whole sardines in olive oil had a vitamin D_3 peak that eluted at 11.9 minutes (Figure 13). This time is similar to the eluted peak for

the standard (Figure 12a). The UV absorption spectrum for the vitamin D_3 peak from olive oil showed a peak at 265 nm and a trough at 228 nm (Figure 14), a characteristic from the standard vitamin D_3 absorption spectrum (Figure 12b). The olive oil extracts were run in triplicate, the chromatograms and UV absorption spectra for each sample were very similar.

The area under the peak was converted to micrograms of vitamin D_3 to determine how much vitamin D_3 was in the sample. The tritium that was collected was counted to determine the starting concentration of vitamin D_3. The vitamin D_3 concentration in the olive oil extracts (three cans) is found in Table 3A. The average vitamin D concentration in the olive oil is found in Table 3B. The results in Table 3B display the average concentration of vitamin D_3 in a 1 mL sample of olive oil and Table 3C displays the average concentration of vitamin D_3 in the olive oil from one can of sardines.

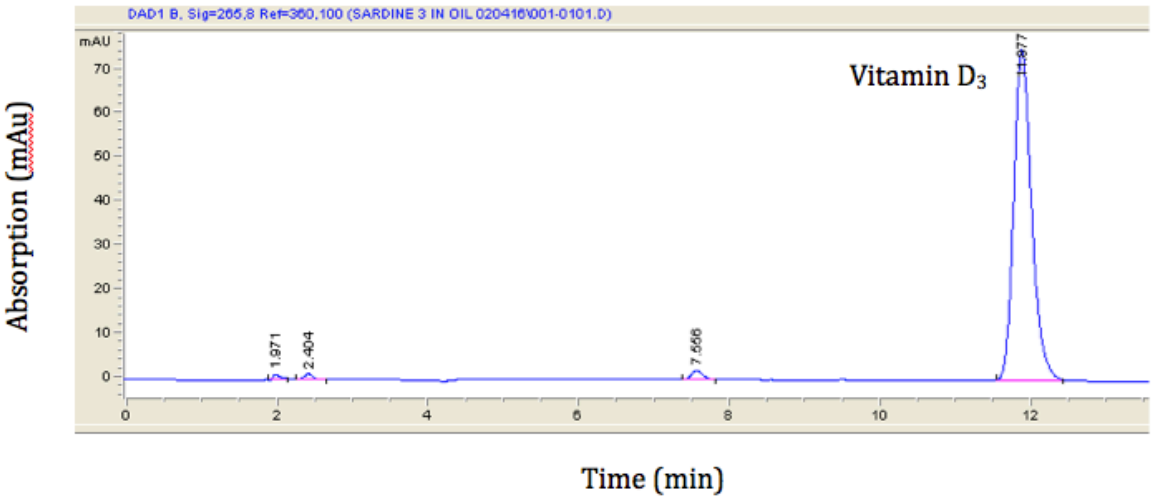

Figure 12a: Control chromatogram of standard vitamin D_3 in 0.8% IPA in hexane on straight phase HPLC detected at 265 nm (prior to running samples of olive oil from can of sardines canned in water).

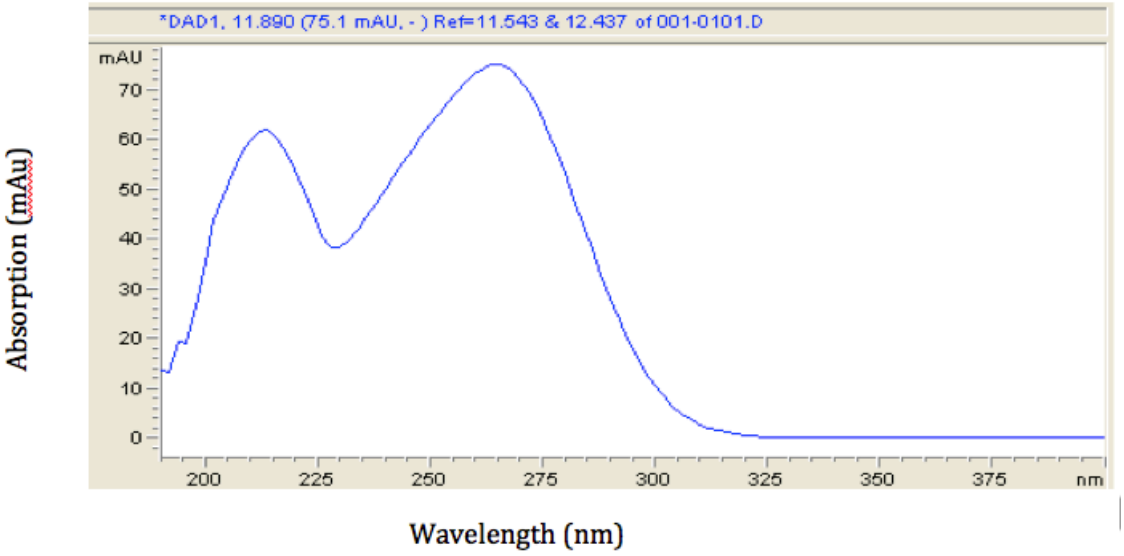

Figure 12b: UV absorption spectrum recorded from the peak that eluted at 11.89 min from figure 12a

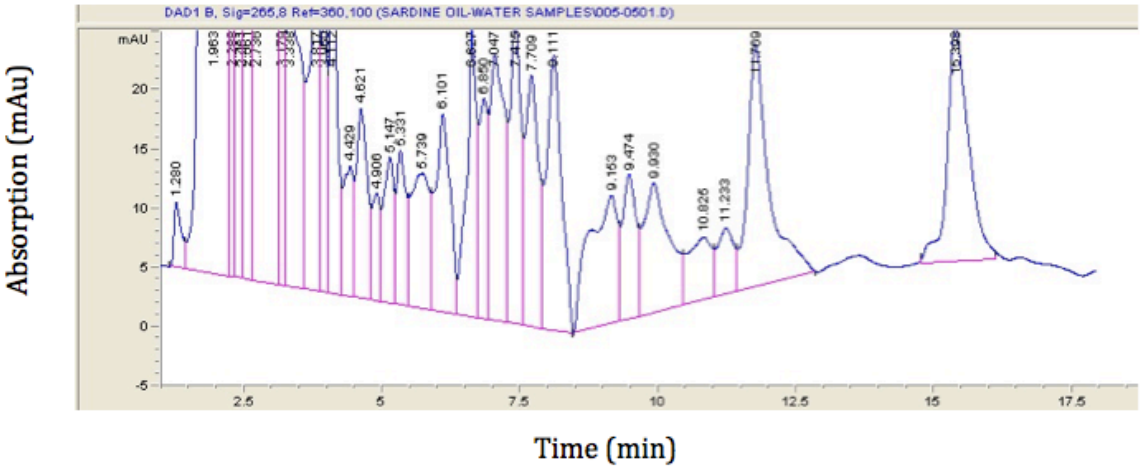

Figure 13: Lipid extract from olive oil from sardine that were canned in olive oil and chromatographed on straight phase HPLC with 140 µL of 0.8% isopropanol in hexane at a flow rate of 1.5 mL/min

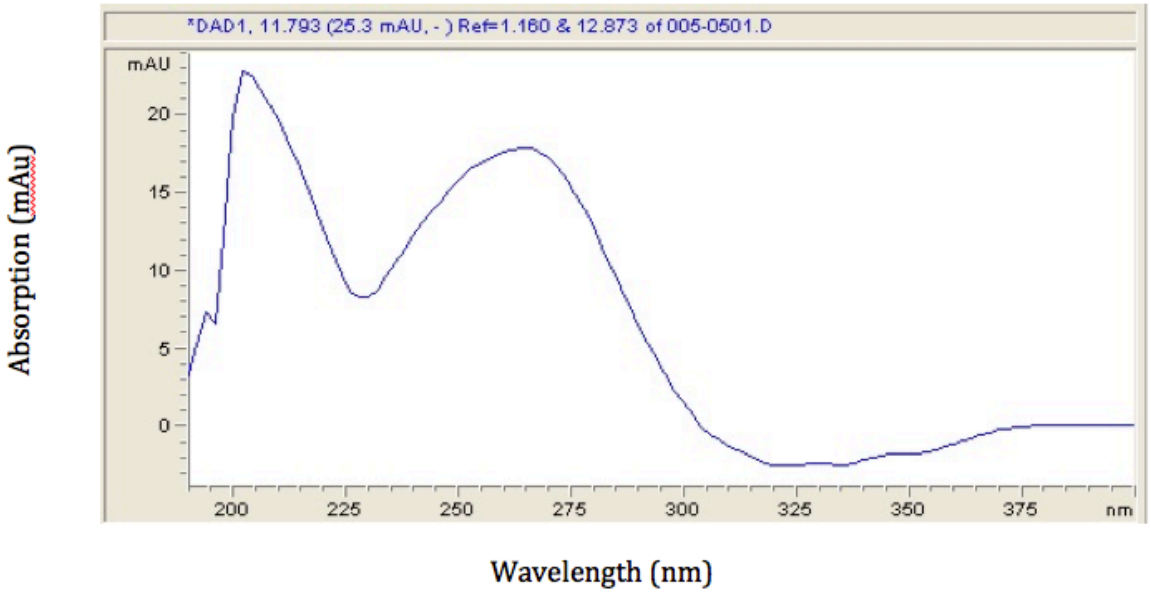

Figure 14: UV absorption spectrum recorded from the peak that eluted at 11.9 min from figure 13

Table 3A: Vitamin D_3 concentrations of 1 mL sample of olive oil from a can of sardines canned in olive oil				
	Volume of Oil (mL)	% Recovery	Vitamin D_3 (µg/mL)	Vitamin D_3 (IU/mL)
Can 1	1.0	32.5	0.69	27.5
	1.0	40.0	0.31	12.5
	1.0	37.5	0.42	17.0
	Mean±SD		**0.47±0.20**	**19.0±7.7**
Can 2	1.0	11.9	2.56	102.3
	1.0	15.0	1.68	67.0
	1.0	10.2	3.97	158.7
	Mean±SD		**2.12±1.16**	**109.3±46.2**
Can 3	1.0	34.0	0.38	15.1
	1.0	38.8	0.46	18.4
	1.0	25.0	0.38	15.3
	Mean±SD		**0.42±0.05**	**16.2±1.8**

Table 3B: Vitamin D_3 content in 1 mL samples of olive oil from three cans of sardines canned in olive oil		
Can	Vitamin D_3 (μg/mL)	Vitamin D_3 (IU/mL)
1	0.47	19.0
2	2.12	109.3
3	0.42	16.2
Average	**1.00±0.97**	**48.2±53.0**

Table 3C: Vitamin D_3 content of total olive oil from three cans of sardines in olive oil			
Can	Volume of total oil in can (mL)	Vitamin D_3 (μg/can)	Vitamin D_3 (IU/can)
1	33.4	15.84	634.6
2	11.0	23.29	1202.3
3	16.5	6.89	267.3
Average	**20.3**	**15.34±8.21**	**701.4±471.1**

In a 1 mL sample of olive oil from sardines canned in olive oil there is an average of 1.00±0.97 μg of vitamin D_3 (48.2±53.0 IU)(Tables 3A–C). The oil in each can was collected and measured, with an average volume of 20.3 mL of oil/can. In each can, an average of 15.34±8.21 μg of vitamin D_3 (701.4±471.1 IU) was present. The standard deviations are very large for this group because in can 2 (Table 3A) the amount of vitamin D_3 that was determined was much higher than what was found in cans 1 and 3.

Vitamin D_3 content in Water from canned sardines in water

Water extracted from a can of sardines canned in water was saponified, extracted, and run through HPLC to determine its vitamin D_3 content. It was found that the water from canned sardines in water had a vitamin D_3 peak that eluted at 10.8

minutes (Figure 16). This time is similar to the eluted peak for the standard D_3 (Figure 15a). The UV absorption spectrum for the water extracts shows a peak at 265 nm and a trough at 230 nm (Figure 17), a characteristic for a UV absorption spectrum of vitamin D_3 (Figure 15b). The water extracts were run in triplicate, the chromatograms and UV absorption spectra for each sample were very similar.

The area under the peak was converted to micrograms of vitamin D_3 to determine how much vitamin D_3 was in the sample. The tritium that was collected was counted to determine the starting concentration of vitamin D_3. The vitamin D_3 concentrations for the water extracts (three cans) can be found in Table 4A. The results in Table 4B show the average concentration of vitamin D_3 in a 1 mL sample of water extract and Table 4C displays average concentrations of vitamin D_3 in 1 can of sardines canned in water.

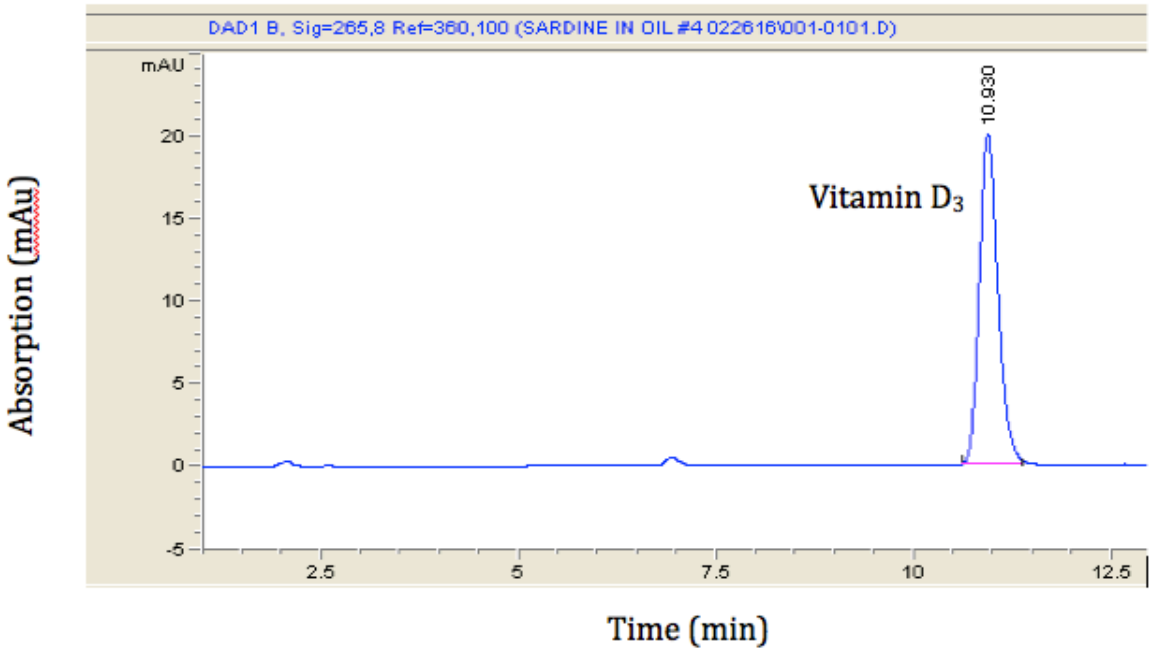

Figure 15a: Control chromatogram of standard vitamin D_3 in 0.8% IPA in hexane on straight phase HPLC detected at 265 nm (prior to samples running of water from can of sardines canned in water).

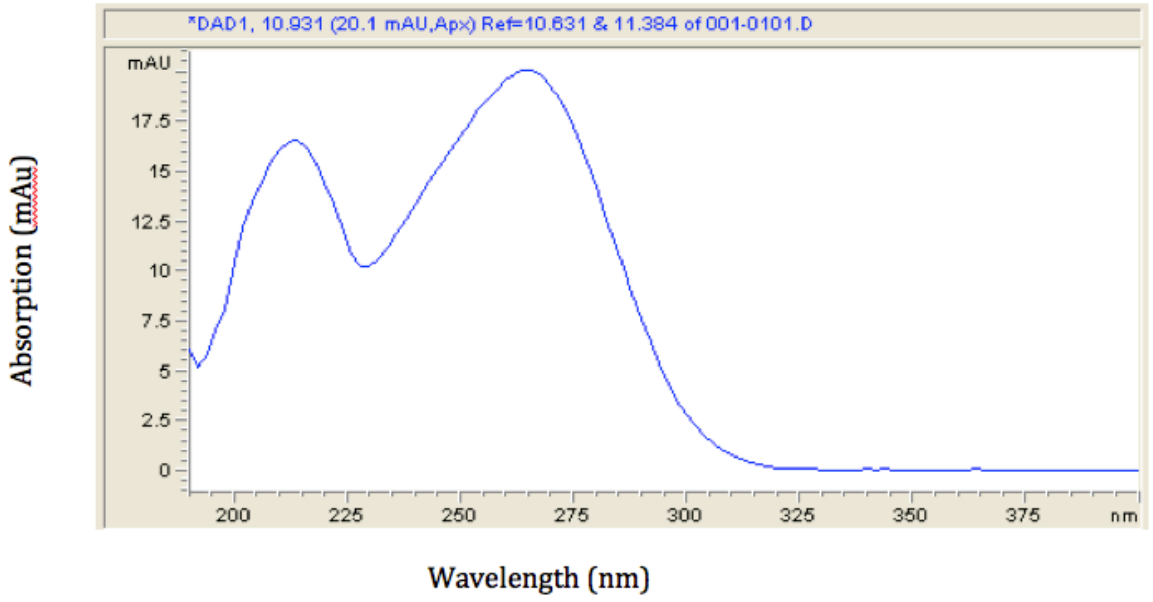

Figure 15b: UV absorption spectrum recorded from the peak that eluted at 10.93 min from figure 15a

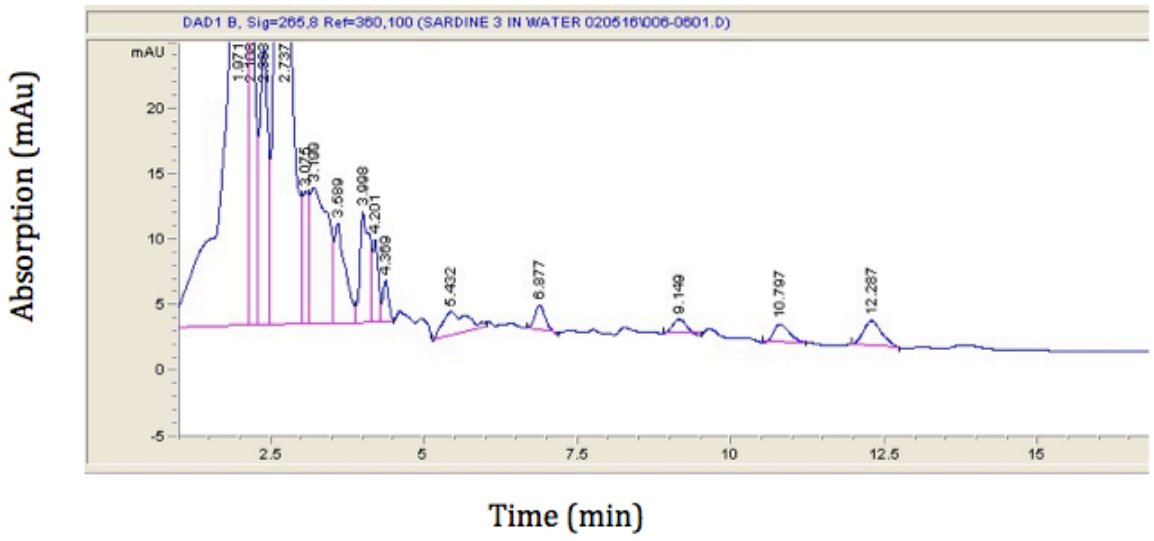

Figure 16: Lipid extract from water from sardine can that was canned in water was chromatographed on straight phase HPLC with 140 µL of 0.8% isopropanol in hexane at a flow rate of 1.5 mL/min

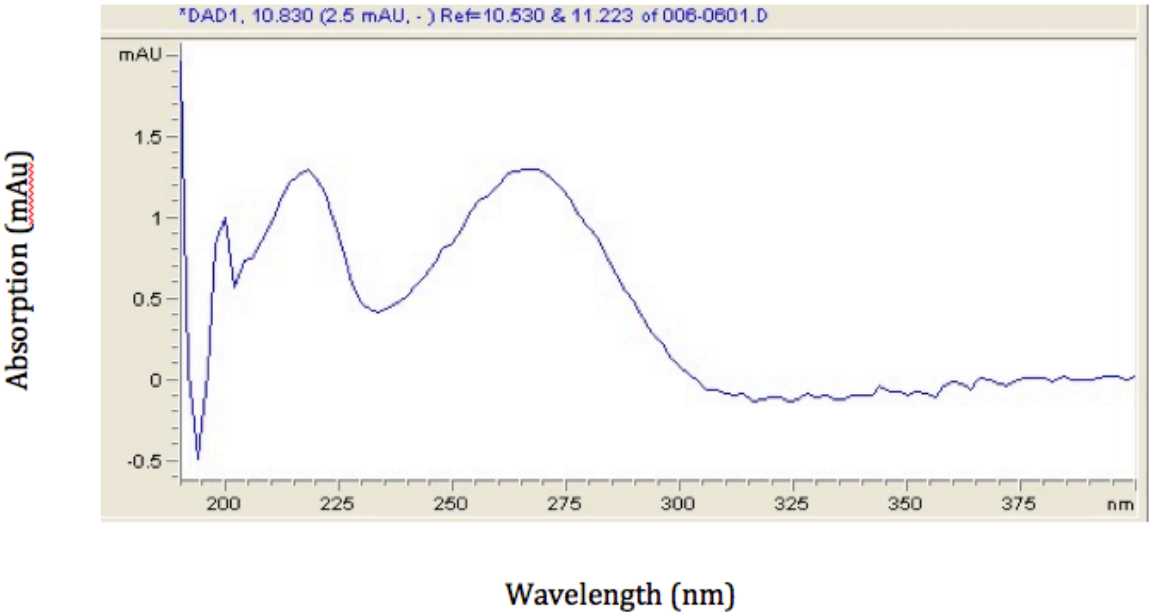

Figure 17: UV absorption spectrum recorded from the peak that eluted at 10.8 min from figure 16

Table 4A: Vitamin D_3 concentrations of 1 mL samples of water from 3 cans of sardines canned in water				
	Volume of Water (mL)	% Recovery	Vitamin D_3 (μg/mL)	Vitamin D_3 (IU/mL)
Can 1	1.0	51.5	0.15	6.0
	1.0	43.3	0.09	3.6
	1.0	53.5	0.05	1.9
	Mean±SD		**0.10±0.05**	**3.8±2.0**
Can 2	1.0	13.2	0.31	12.4
	1.0	12.4	0.08	3.3
	1.0	0.0	0.00	0.0
	Mean±SD		**0.20±0.16**	**5.2±6.4**
Can 3	1.0	26.2	0.07	2.9
	1.0	28.5	1.20	48.1
	1.0	252	0.18	7.1
	Mean±SD		**0.64±0.62**	**19.4±25.0**

Table 4B: Vitamin D_3 content in 1 mL water extract samples from three cans of whole sardines canned in water		
Can	Vitamin D_3 (μg/mL)	Vitamin D_3 (IU/mL)
1	0.10	3.8
2	0.20	5.2
3	0.64	19.4
Mean±SD	**0.31±0.29**	**9.5±8.6**

Table 4C: Vitamin D_3 content of total water from three cans of sardines canned in water			
Can	Volume of total water in can (mL)	Vitamin D_3 (μg/can)	Vitamin D_3 (IU/can)
1	29.0	2.78	110.2
2	27.5	5.39	143.0
3	10	6.38	194
Mean±SD	**22.2**	**4.85±1.86**	**149.1±42.2**

In a 1 mL sample of water from sardines canned in water there was an average of 0.31±0.29 μg of vitamin D_3 (9.5±8.6 IU)(Tables 4A–C). The water in each can was collected and measured, with an average volume of 22.2 mL of water/can. In each

can, an average of 4.85±1.86 μg of vitamin D_3 (149.1.4±42.2 IU) was present. The standard deviations are large for this group because the values obtained in the second can were much higher than cans 1 and 3.

Comparisons of all vitamin D_3 content in sardines and their canned solutions

Table 5: Comparison of total vitamin D_3 concentrations in a can of sardines

Group	Vitamin D_3 (μg/can)	Vitamin D_3 (IU/can)
Sardines canned in Olive Oil	63.85±5.92	2,555.6±234.2
Olive oil	15.34±8.21	701.4±471.1
Sardines canned in water	49.79±60.33	1,993.7±2,411.3
Water	4.85±1.86	149.1±42.2

Sardines canned in olive oil had the highest concentration of vitamin D_3 at 63.85±5.92 μg of vitamin D_3. However, the concentration of vitamin D_3 in sardines in oil is 1.28 times greater ($p>0.05$) than sardines canned in water. When comparing the solutions, the olive oil had more than 4.7 times the vitamin D_3 than water. Figure 18 shows all samples of sardines (both in oil and non-oil solutions).

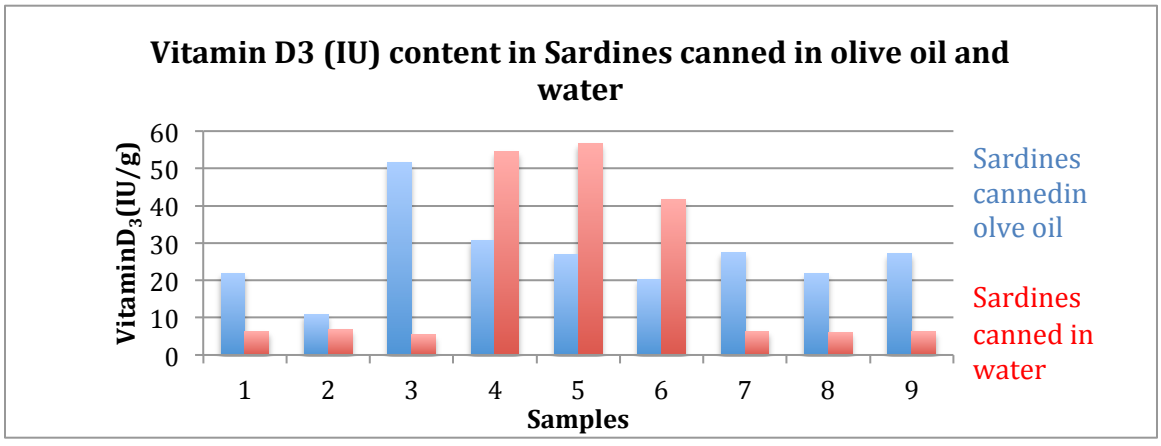

Figure 18: Vitamin D_3 (IU) content in sardines in both olive oil and water

There was more vitamin D_3 in sardines canned in olive oil, however in can 2 of sardines in water there was a larger amount of vitamin D_3. It is unclear if there was an error in the analysis of the fish content making the vitamin D content higher than it actually is. Figure 19 shows the comparison of vitamin D_3 in both cans, sardines in oil and sardines in water.

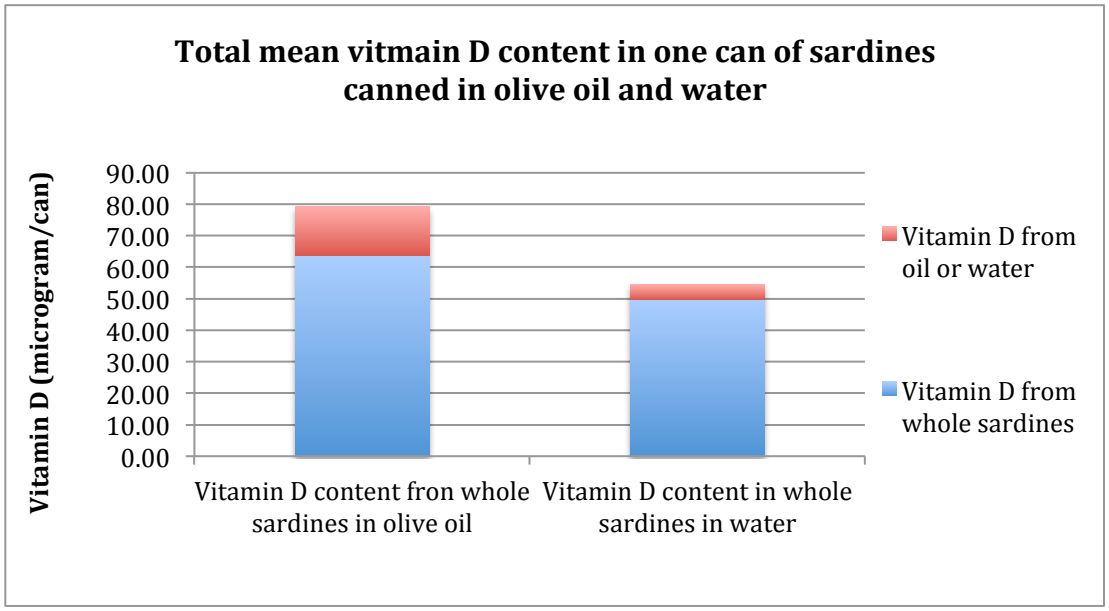

Figure 19: Vitamin D_3 in cans of sardines in oil and water

While it appears that there is more vitamin D_3 in a can of sardines canned in oil, the proportion of vitamin D_3 from just the sardines is greater in sardines canned in water. Sardines canned in water had 85.8%±10.4% of the total vitamin D content in the can compared to sardines canned in oil, where only 79.1%±12.8% of the total vitamin content in the can was present in the sardines.

DISCUSSION

According to the US Department of Agriculture (USDA) 100g of whole sardines canned in olive oil contains 193 IU of vitamin D_3. Byrdwell et al. reported that canned sardines had 145 IU/100 g of sardine.[45] O'toole and Holick, reported that 100g of canned sardines in olive oil (Manufactured by Chicken of the Sea) had 330.8 IUs of vitamin D_3.[46] An evaluation of fresh sardines found that they contained 250 IUs in a serving of 106.3 g.[47]

In this study, 1 can of sardines in olive oil, which contained approximately 3 sardines with a total average weight of 96 grams, had 2,555.6±234.2 IUs of vitamin D_3. Sardines submersed in water had 1,993.7±2,411.3 IU's of vitamin D_3 per can of sardines.

Little was known if the medium that the sardines are canned in has any influence on the sardines' vitamin D content. An analysis of the oil revealed that there was 701.4±471.1 IU's of vitamin D_3 while the water contained 149.1±42.2 IUs. The results support the concept that the oil was extracting some of the fat-soluble vitamin D from the sardines. It was hypothesized that sardines packed in water might contain more vitamin D and less would be found in the water. When comparing vitamin D content in sardines from oil and water, there is more vitamin D_3 in sardines canned in oil compared to water. However, the fraction of vitamin D content in sardines canned in oil compared to water was 79.1%±12.8% and 85.8±10.4% ($p<0.05$), respectively. Thus the hypothesis that there is less vitamin D

extracted from sardines canned in water appeared to be correct. An evaluation of the vitamin D_3 content in the olive oil and water used to can the sardines revealed 701.4±471.1 and 149.1±42.2 IUs in total olive oil and water respectively. It was determined that of the total vitamin D content in the can (sardines in olive oil or water) 20.9%±12.8% of vitamin D_3 is found in the olive oil compared to only 14.2%±10.4% ($p<0.05$) vitamin D_3 found in water. These results support the concept that sardines packed in olive oil will have less vitamin D_3 than similar sardines packed in water.

In comparison to some of the more well-known fish high in vitamin D content, like salmon (1320 IUs/100g)[45], the canned sardines that were evaluated had an even greater vitamin D_3 content. It was determined that in a 100g sample, Vital Choice sardines canned in olive oil had 13.2 times greater vitamin D content compared to the values reported by the USDA. Likewise, when comparing sardines produced by Chicken of the Sea, it was determined that the Vital Choice sardines canned in olive oil had 7.7 times the vitamin D content. The type of sardine and the season that they were obtained could explain this variability. All sardines from Vital Choice, the supplier of the canned fish used in this study, are from the family *sardina* found in Portugal and caught during the summer months. Vital Choice prides themselves on the freshness of their sardines. As previously mentioned, the sardines are immediately placed on ice after extraction from the ocean and are then immediately canned upon arrival on shore. This process preserves the freshness of the sardines and could potentially explain the high vitamin D content as an accurate

indicator of original sardine vitamin D content.

There are four species of sardines: *Sardina, Sardinops, Sardinella,* and *Dussumieria.*[48] All four species come from varying parts of the world: Australia, South America, South Africa, Japan, Europe, India, and California. Thus, having sardines from one population in Portugal does not permit generalization about sardines from the rest of the world. Also, the time at which the sardines were caught has a great influence on the vitamin D content in the sardines. Comparing this study with other known data is challenging, as it is incorrect to assume that sardines from other studies were from the same geographic location and gathered at the same time. This variability in geographical location of sardine retrieval could possibly explain the differences in determined vitamin content in a sample of sardines. For example, sardines gathered in the winter months would naturally have less vitamin D than those gathered in the summer months due to their food source's (local zooplankton and phytoplankton) inability to generate vitamin D from the unusable UVB rays. Sardines that live at lower depths will also have lower vitamin D levels than those at more shallow depths due to the scarcity of phytoplankton with lower vitamin D content.

There were a few outliers generated in this study that need to be accounted for, in particular the sardines canned in water and the water itself. The radioactive recovery for those samples was very poor, some less than 10% recovery. This low recovery assumes that that vitamin D content at the beginning of the study was much greater than what was counted at the end. This assumption generated an

abnormally large value of vitamin D content and could explain the dissimilar values found in that sample.

Areas of future research should include an analysis of canned sardines from different manufacturers to account for variation in types of sardines from around the world. An analysis of other canned fish in liquid suspensions would also help draw conclusions on the effect of submersion of fish in oil and the extraction of vitamin D from fish to the canned medium. Finally, a comparative analysis of vitamin D concentrations in canned fish versus fresh fish would help our understanding of the effect of canning on vitamin D content in canned fish.

For people who are vitamin D deficient, including sardines into the diet can help increase improve the vitamin D status. For example, according to the Endocrine Society's guidelines, if someone is vitamin D deficient, (s)he will need to supplement between 1500 and 2000 IUs of vitamin D each day. Consuming a can of Vital Choice sardines will provide a sufficient amount of vitamin D to help satisfy this requirement. One should also note the nutritive benefit in the olive oil in canned sardines. Thus, it would be beneficial to also consume the olive oil as it also contains vitamin D.

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CURRICULUM VITAE

Tyler Kalajian | ty.kalajian@gmail.com **|** YOB: 1992

Education

Boston University | *2014*
Bachelor of Science in Human Physiology, *Cum Laude*
Minor in Speech, Language, and Hearing Sciences

Boston University School of Medicine | *May, 2016*
Division of Graduate Medical Sciences
Masters in Medical Sciences

Personal Experience

Emergency Medical Technician at BU EMS | *Fall 2011 – December 2014*
Assisted university patrons when emergency medical care is needed
Provide a safe and comfortable environment for patrons at BU
Attend continuing education to maintain up-to-date knowledge on various emergency medical techniques and procedures

Intern for Orthopedic Surgeon, Gregory Tchejeyan | *Summer 2011*
Observed various orthopedic procedures and how they work
Eagerly participated in patient contact and confrontation
Learned how a doctor balances his work life with family life

Research

Clinical Research Coordinator, Vitamin D, Skin, and Bone Research Laboratory Fall 2015–Present
Orchestrated studies in the Endocrinology department and actively enrolled patients
Worked closely with the Boston University Institutional Review Board to develop studies and amend current studies
Actively engaged in basic research laboratory experiments to better understand the nature of vitamin D

Research Assistant, Department of Radiology at Boston Medical | Fall 2014–Winter 2015
Extracted information from patient medical records
Work with fellow doctors in the analysis of x-rays and CT scans of various pelvic injuries
Taught and used REDCap, a data entering service, for the compilation of the extracted information

Research Assistant, Orthopedic Hand Service at Mass General | *Spring 2014*
Enrolled patients into various orthopedic research studies in the clinic
Carried out follow-up phone calls to all patients enrolled in studies
Worked with the fellow PhD and MD students to produce new informative studies
Assisted the PhD and MD students in the write up and submission of studies

Research Assistant, Department of Radiology at Boston Medical | *Fall 2012–Winter 2014*
Assisted and published in the research and composition of research regarding various techniques to detect sickle cell disease in pediatric patients.
Analyzed over 200 patient profiles extracting information regarding their sickle cases
Learned how to analyze cat scans and radiographs of specifically the kidneys and spleen to observe dysfunctions and deformities that could indicate early onset of Sickle Cell Disease

Activities

Volunteer with Birthright Armenia – *Summer 2014*
Lived/traveled in Armenia for two months spending time volunteering for nonprofits (see below)
Traveled to many rural communities where I participated in many community service projects whether it was building parks, homes, or cleaning up the community
Took classes to perfect my Armenian Language to better my communication skills
Attended conferences and lectures regarding various current events in Armenia (Political, Economical, Medical, Technological, etc.)

Medical Intern at Plastic Surgery Department – Yerevan State Medical University – *Summer 2014*
Observed plastic and Microsurgery procedures
Assisted with pre operational and post operational procedures
Comforted patients and families post operation
Attended a National medical conference where I was able to observe advancements in the Armenian Medical Sector

Devised a curriculum for participants of varying mental and physical capabilities in the program
Taught the teachers some American activities they could use with their students
Observed components lacking in the current curriculum to relay back to the states to assist with funds

President for Armenian Student Association | *January 2013 – January 2014*
Organize many fund raising events to benefit local charities
Worked diligently to foster Armenian youth relationships in the Greater Boston area
Maintained a strong network between a dozen other Armenian Students Associations in the Boston area by organizing collaborative events and lectures

Executive Board for Boston University Student Union | *Fall 2010*
Represented the BU student body by voicing the students problems and concerns to the student government and administration
Worked 10 hours a week doing various activities to strengthen the student government

Member of Premedical Society | *Fall 2010 – May 2014*
Attended many medical programs at BU, where medical professionals from all over the Boston area would come and present about their practice
Eagerly attained much knowledge regarding the medical atmosphere as well as gained insight to the Medical School application process.

Medical Brigade to Honduras | *May 2013*
Improve health care access in rural communities
Offer comprehensive consolation services for members of the Honduran communities
Educate local Hondurans on various public health issues

Peer Counselor, Sargent College | 2013-2014 Academic Year
Will act as a counselor for incoming freshman to Sargent College by helping them improve study skills, learn how to get involved in activities around campus, and help them transition from high school to a college setting.

I also act as a Counselor for external transfer students. I help get the students acquainted with BU and the city and foster a strong relationship with them to reassure to them that they always have someone who they can rely on.

College Outreach Coordinator for Peter Koutoujian Campaign | *Summer–Fall 2013*
Act as a liaison between the Campaign for Peter Koutoujian (running for US Congress) and college students.
Assist in phone banking, canvassing, and organizing awareness events for the campaign.

Publications

Gale, H., Setty, B., Sprinz, P., Doros, G., Williams, D., Morrison, T., … Castro-Aragon, I. (n.d.). Implications of Radiologic-Pathologic Correlation for Gallbladder Disease in Children And Young Adults with Sickle Cell Disease. *Emergency Radiology*. (Publish date June 2015)

Menendez, M. E., Thornton, E., Kent, S., Kalajian, T., & Ring, D. (2015). A prospective randomized clinical trial of prescription of full-time versus as-desired splint wear for de Quervain tendinopathy. *International Orthopaedics*. http://doi.org/10.1007/s00264-015-2779-6

Languages

Fluent in English
Conversant in Armenian and Spanish

Awards

Deans List Fall 2012 (obtaining GPA greater than a 3.5)
Deans List Spring 2014
Scarlet Key Honors Society | Inducted *October 2013*
The Dean of Students presents this high honor to graduating seniors each year as part of a continuing effort to recognize those who have made notable contributions to Boston University through their distinguished accomplishments.

Interests

Musculoskeletal system
Orthopedics
Otolaryngology
Dietetics and Health
COPD and Respiratory disorders