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Evaluating the vitamin D content in sardines and mackerel

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EVALUATING THE VITAMIN D CONTENT IN
SARDINES AND MACKEREL

by

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DEDICATION

I would like to dedicate this work to Lourdes Hormillosa
ACKNOWLEDGMENTS

I would like to thank Dr. Michael F. Holick, for giving me this great opportunity to conduct research in his vitamin D lab and allowing me to learn about his passion of vitamin D.

I would also like to thank my advisor, Dr. Carl Franzblau, for his guidance and support since the start of my enrollment in the Medical Sciences program.
EVALUATING THE VITAMIN D CONTENT IN
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PATRICK O'TOOLE

ABSTRACT

Vitamin D is an important secosteroid hormone that is responsible for calcium and phosphorus homeostasis. Vitamin D deficiency and insufficiency are an ever increasing global problem. Very few foods naturally contain vitamin D; such as salmon, and sundried or ultraviolet irradiated mushrooms. Few foods are fortified with vitamin D such as milk, orange juice, cereal and bread. Little is known about the vitamin D levels in certain fish such as sardines. The purpose of this study was to find out whether sardines and mackerel are a good source of vitamin D such as wild salmon. It was hypothesized that both sardines and mackerel are a good source of vitamin D. Based on the results, sardines are a good source of vitamin D. One serving size (3.5 ounces, about 5 fish) of sardines has about 330.8 IU’s of vitamin D₃. This is equal to 66.2 IU’s of vitamin D₃ per fish. Mackerel on the other hand does not have as much vitamin D₃ as sardines. A standard serving of mackerel (3.5 ounces, about 3 fish) has 81.6 IU’s of vitamin D₃. This is approximately 27.2 IU’s of vitamin D₃ per fish. Both mackerel and sardines are good sources of vitamin D₃.
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\(23, 24, 25, 26, 27, 28, 29, xi\)
265nm

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LIST OF ABBREVIATIONS

1,25(OH)D…………………………………………………………1,25-dihydroxyvitamin D
25(OH)D…………………………………………………………25-hydroxyvitamin D
7-DHC…………………………………………………………7-dehydrocholesterol
ACN………………………………………………………Acetonitrile
HPLC……………………………………High Performance Liquid Chromatography
Kg………………………………………………………………Kilogram
IOM……………………………………………………………Institute of Medicine
IU………………………………………………………………International Units
MeOH…………………………………………………………Methanol
RHPLC………………………………………………………Reverse Phase HPLC
Vitamin D$_2$……………………………………………………Ergocalciferol
Vitamin D$_3$……………………………………………………Cholecalciferol
VDR…………………………………………………………Vitamin D Receptor
INTRODUCTION

Background

Vitamin D is a steroid hormone that has been around for more than 500 million years, first produced by phytoplankton in the form of vitamin D$_2$.\textsuperscript{1} Here we are today, as complex organisms, still elucidating the importance of vitamin D in our bodies. History and epidemiology have shown the importance of vitamin D.

Vitamin D’s first major footprint in history and public health was during the industrial revolution in Europe. During the 1600’s with industrialization of cities came severe air pollution. It was also during this time in which many children developed a severe disease called rickets; characterized by bone deformities and muscle weakness.\textsuperscript{1,2} On the other hand, children living in rural, non-polluted-areas did not develop rickets.\textsuperscript{1} It was discovered in the early 1900s, that children exposed to a mercury arc lamp experienced improvement in their symptoms.\textsuperscript{3} It eventually became clear that vitamin D was pivotal in helping resolve rickets. Eventually many foods became fortified with vitamin D and cod-liver oil pills became a popular means of treating and preventing rickets.\textsuperscript{2,4}

There are two major forms of vitamin D and they are vitamin D$_3$ (cholecalciferol) and vitamin D$_2$ (ergocalciferol). D$_3$ is produced in the skin from 7-dehydrocholesterol (7-DHC) and vitamin D$_2$ is produced in yeast and certain plants from ergosterol.\textsuperscript{5} 7-DHC can be considered the provitamin D$_3$ and ergosterol can be considered the provitamin D$_2$. The difference between vitamin D$_3$ and vitamin D$_2$ is that the side chain for vitamin D$_2$ contains a double bond
between C-22 and C-23 and a methyl group on C-24. In regards to dietary supplementation, it has been shown that vitamin D$_2$ is as effective as vitamin D$_3$ in maintaining vitamin D homeostasis. Both vitamin D$_2$ and vitamin D$_3$ are available as over the counter supplements. But only vitamin D$_2$ is available as a pharmaceutical agent in the United States.

![Figure 1. Chemical Structures of 7-DHC, Ergosterol, Vitamin D3, and Vitamin D2. Copyright MacLaughlin and Holick (1983) Reproduced with permission.](image)

**Vitamin D in Phytoplankton**

In 1925 it was hypothesized that vitamin D in cod liver oil came from light hitting green plankton. Plankton produce over 120 billion tons of organic carbon
per year compared to 20 tons produced by terrestrial plants, and throughout the food chain in ocean, the higher organisms such as whales eat a proportionally higher amount of plankton than a fish such as a sardine or mackerel.\textsuperscript{10,11} In 1982 a study was done on cultures of \textit{Skeletonema menzelii} and \textit{Emiliania huxleyi}, (a clone of BT-6) which is an ancient phytoplankton that is believed to have existed for at least 500 million years, to see if either of those two types of phytoplankton are able to produce vitamin D.\textsuperscript{12} The organisms that were cultured in the absence of UVB radiation; HPLC analysis showed that ergosterol (provitamin D\textsubscript{2}) was present.\textsuperscript{12} The phytoplankton that were exposed to simulated sunlight; during their HPLC analysis, there was less provitamin D\textsubscript{2} present and high amounts of previtamin D\textsubscript{2}.\textsuperscript{12} Phytoplankton and zooplankton can make vitamin D\textsubscript{2} and can contribute this to the food chain.\textsuperscript{10}

\textbf{Production in Humans}

Vitamin D is a secosteroid hormone that is produced endogenously in humans. Vitamin D synthesis occurs during exposure to sunlight, ultraviolet B (UVB) radiation; 290-315nm.\textsuperscript{13} 7-DHC which is located in the epidermis and dermis absorbs UVB radiation and gets converted to previtamin D\textsubscript{3}. It is in the plasma membrane of the skin cells where previtamin D\textsubscript{3} is converted to vitamin D\textsubscript{3}. Ultimately vitamin D\textsubscript{3} travels to the liver and is then absorbed in the gastrointestinal tract and enters the lymphatic system.
The major circulating form of vitamin D is metabolized to 25-hydroxyvitamin D (25(OH)D), and it is that same metabolite that is used to assess a person’s vitamin D status. While 25(OH)D is the major circulating form, it is not the biologically active form, and that is 1,25-dihydroxyvitamin D (1,25(OH)D). 13 25(OH)D has a circulating half-life of 2-3 weeks, whereas 1,25(OH)D has a half-life of 4 hours. 14 The Earth’s axis effects the angle at which the sun’s UV rays enter the atmosphere. During the summer, more vitamin D is made. During the winter time much of the UVB radiation from the sun is absorbed by the ozone layer. 5 Since sunlight is the major source of Vitamin D for humans, seasonal variation causes corresponding variation in blood serum levels of 25(OH)D. 15 At the end of a winter in the New England region of the United States, Caucasian adults typically have a blood level of 25(OH)D between 18-22ng/mL. 7 During the same period of time, African American adults had a level between 13-15ng/mL. 7 Therefore, vitamin D deficiency is certainly a problem that can manifest itself during that time period and skin color is a factor too in vitamin D production. 16

Many people may now actively avoid the sun or use sunscreen to decrease their risk for skin cancer. This avoidance of sun exposure or use of sunscreen can cause a decrease in Vitamin D levels. Unfortunately many diets are poor in vitamin D. Few foods such as oily fish (wild salmon) have high levels of vitamin D but ultimately there are relatively few sources of fortified foods on the market. 16
Extracting Vitamin D:

Vitamin D is a lipophilic molecule and since it is fat soluble saponification is necessary to hydrolyze triglycerides into glycerol and fatty acids.\textsuperscript{17} Natural food sources such as fish contain a high content of lipid soluble molecules including triglycerides, this makes it difficult to run direct directing for vitamin D. The gold standard of analyzing the vitamin D content of certain foods involves the use of High Performance Liquid Chromatography (HPLC) followed the utilization of mass spectrometry or a photo diode variable wavelength detector.\textsuperscript{17,18} Using a reverse phase column allows one to better distinguish between compounds of similar polarity. This is important because it allows the use of an internal standard such as vitamin D\textsubscript{2}. Two popular options for analyzing vitamin D on a reverse phase HPLC is using either a C-18 or a C-30 column.\textsuperscript{18} Along with using saponification and HPLC, the use of an internal standard is a way to determine the recovery which is then used to determine the concentration of vitamin D in a sample.

Vitamin D in Fish

Vitamin D is produced in the form of vitamin D\textsubscript{3} in fish and some species of fish contain much higher concentrations of vitamin D\textsubscript{3} in their liver or soft tissues compared to terrestrial vertebrates.\textsuperscript{19} Since vitamin D is fat soluble, it is
found in fat storage areas in fish such as the head, viscera, liver and muscle tissue. Recall that for humans and other land dwelling animals, vitamin D₃ is produced when 7-DHC is irradiated with UVB radiation. The problem for fish is that UVB radiation can’t penetrate very far down into the ocean. In sea water, UVB radiation is absorbed within the first few centimeters. Thus fish likely obtain vitamin D₃ from the food chain.

In one published study, researchers analyzed lamprey eels, skipjack tuna, and albacore and in doing so they compared the vitamin D content in the skin, liver, alimentary canal, and flesh. It was found that most of the vitamin D was found in the liver. Skipjack tuna and albacore had 41,240 ng/g and 21,000 ng/g of vitamin D₃, respectively for liver content of vitamin D₃. In comparison, the skin of skipjack tuna and albacore had 454 ng/g and 257 ng/g of vitamin D₃, respectively. Interestingly, the 7-DHC levels in the skin for the skipjack tuna and albacore were both very low, only 101ng/g for both. They concluded that the high levels of vitamin D₃ in the liver are attributed to biosynthetic pathways or direct ingestion of vitamin D₃ and not conventional photochemical synthesis with UVB radiation in the fish’s skin.

Researchers studied rainbow trout that were reared from eggs in darkness and once hatched; they were fed a vitamin D-free diet. Their study involved the use of radiolabelled D₃, ultraviolet A (350-400nm) (UVA), UVB, and blue visible light (380-480nm). They found that when vitamin D- deficient trout were injected with ³H-Vitamin D₃, 90% of the injected D3 wound up as bile rather than being
deposited in the kidneys, liver or ending up in plasma.\textsuperscript{20} However, when the researchers fed \textsuperscript{3}H=Vitamin D\textsubscript{3} to other vitamin D deficient rainbow trout, and they noted that most of that D\textsubscript{3} wound up in the liver as a fatty acid ester. Furthermore, rainbow trout that were reared for 2 years in darkness and exposed them to a 60 watt incandescent light bulb for 24 hours; this lead to the death of all the fish due to hypercalcemia.\textsuperscript{20} They concluded that in vitamin D- deficient states the 1-hydroxylase which is responsible for forming the active form of vitamin D; became very active, they postulated that this resulted in high levels of 1,25(OH)D and finally hypercalcemia.\textsuperscript{20} Ultimately, they found it possible for the 7-DHC in the skin of rainbow trout to be converted to D\textsubscript{3} under blue light (380-480nm) which can penetrate 200 meters of water.\textsuperscript{20} This can perhaps be a nonconventional means in which some fish species produce vitamin D.

One study conducted by a group of researchers in Canada investigated the role of vitamin D in rainbow trout physiology. They found that rainbow trout that were fed a diet devoid of vitamin D for 6 months showed a 56\% incidence of muscle tetany.\textsuperscript{21} Incidentally, when the fish were administered vitamin D\textsubscript{2} or vitamin D\textsubscript{3}, the symptoms decreased the incidence in a linear manner.\textsuperscript{21} Furthermore, electron microscope analysis revealed that white muscle fibers were adversely affected by the deficiency in vitamin D.\textsuperscript{21} In another experiment those same researchers fed other groups of rainbow trout certain amounts of D\textsubscript{3}; either 200 IU per kg, 400 IU per kg or 800 IU per kg. The 200 IU per kg and 400 IU per kg resulted in fish that had increased liver lipid and increased total lipid
content.\textsuperscript{21} On the other hand, the dosing of 800 IU per kg resulted in decreased liver and total lipid content.\textsuperscript{21} Additionally, fish that were given the higher levels of vitamin D had higher linear increases in growth.\textsuperscript{21} So based off of their results, vitamin D in fish (at least in rainbow trout) may play a role in the proper functioning of muscle tissue, growth and the storage of fat.

In a similar study, researchers fed channel catfish fingerlings 9 different types of experimental diets, which varied in vitamin D content, calcium, and phosphorus.\textsuperscript{22} The fish that were fed the diet that was lacking in vitamin D, gained significantly much less weight and lower percentage of body calcium and phosphorous.\textsuperscript{22}

In regards to vitamin D metabolism, it does appear that certain fish species such as sole, goldfish and lungfish do form 25(OH)D. Researchers conducted that experiment was by using a 25(OH)D binding protein.\textsuperscript{23} Further analysis of other fish species by the same researchers found that fish with cartilaginous skeleton use lipoproteins for binding 25(OH)D$_3$ and it’s metabolites while bony fish had an alpha-globulin transport protein.\textsuperscript{23} In order to find out what organ in a fish’s body that metabolized 25(OH)D$_3$ to 1,25(OH)$_2$D$_3$, a group a researchers took vitamin D deficient trout and exposed them to $^3$H-25(OH)D$_3$. In an absence of calcium, they noticed that the trout produced more 1,25(OH)$_2$D$_3$.\textsuperscript{24} Upon organ analysis of the liver, gills, kidney, gut, and scales; they found that $^3$H-25(OH)D$_3$ was only metabolized in the liver.\textsuperscript{24} Those researchers concluded that the liver in trout can convert 25(OH)D$_3$ to 1,25(OH)$_2$D$_3$ and that calcium
levels in the environment can affect this process. One research team investigated the effects of 1,25(OH)$_2$D$_3$ in tilapia. They found that 1,25(OH)$_2$D$_3$ increases intestinal calcium absorption.

So while there is much variability and some uncertainty between fish species on the importance of vitamin D, it can certainly be said that vitamin D undoubtedly plays a role in the metabolism and physiology in certain fish species.

**Quantifying Vitamin D**

Vitamin D can be quantified in various units. 1 nanogram is equivalent to 2.5 nanomoles. 1 microgram is equivalent to 40 International Units (IU). 1 nanogram/milliliter is equivalent to 2.5 nanomoles per liter. And 1 IU is equal to 25ng.

**Food Sources of Vitamin D**

Few foods naturally contain vitamin D (either D$_2$ or D$_3$). Oily fish are a good source of vitamin D. For instance fresh, wild salmon (3.5oz) has between 600-1000 IU of vitamin D$_3$. Canned tuna (3.6oz) has about 230 IU of vitamin D$_3$. There are some non-fish sources of vitamin D on the market and they are 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$_3$. There
are some fortified foods on the market that also contain vitamin D. fortified milk and fortified orange juice each has about 100IU / 8 oz of vitamin D₃.²⁷

**Prevalence:**

Throughout the literature it is very evident at how prevalent vitamin D insufficiency and deficiency are. According to the Centers for Disease Control and Prevention (CDC), 9%–11% of children aged 1–8 years, 19%–22% of children aged 9–13 years, 22% of children aged 14–18 years, and 22%–28% of adults are vitamin D deficient (serum 25(OH)D below 20ng/mL).²⁸ In a similar study conducted in India involving 5137 children between 10-18 years of age; the prevalence of vitamin D deficiency was 10.4% for boys and 11.1% for girls.²⁹ A study conducted in Romania involving 123 postmenopausal women found that of the cohort, 74.8% had vitamin D deficiency and 17.1% had insufficiency according to ES’s guidelines.³⁰ A similar study conducted in the United States involving 1526 postmenopausal women receiving treatment for osteoporosis, found that 52% of those women had inadequate levels of vitamin D (less than 30ng/mL).³¹ As you can see vitamin D deficiency and insufficiency are very prevalent around the world and should be considered a public health concern.
Function and Health Implications:

One of the main functions of Vitamin D is to maintain serum phosphorus and calcium levels.³² On a cellular level, vitamin D interacts with the vitamin D receptor (VDR) and can affect transcription regulation and bone metabolism.¹⁵ Low levels of vitamin D can lead to alterations in calcium and phosphorus homeostasis, secondary hyperparathyroidism, bone loss, and an increase in fracture risk.³²

There appears to be many health implications of vitamin D on one’s health. During pregnancy there is a correlation between vitamin D insufficiency and low birth weight.³³ Maternal Vitamin D levels appear to be important in the outcome and growth of the fetus. During adulthood vitamin D plays a vital role in maintaining bone mineral density.³³ Some of the ailments that are associated with vitamin D deficiency in adulthood as osteoporosis and osteopenia.³³
Recommended Daily Amounts of Vitamin D

There are differences between the Institute of Medicine’s (IOM) guidelines for vitamin D intake and the Endocrine Society’s guidelines. For children between 1–8 years old the IOM recommends 600 IU or 15 micrograms per day. For males and females between 9–70 years of age, the recommendation from the IOM is still 600 IU or 15 micrograms per day. For adults over 70 of age the IOM recommends 800 IU or 20 micrograms per day. In regards to sufficiency and deficiency, the IOM considers a serum level of 25(OH)D below 50 nmol/L or 20
ng/mL to be deficient and a serum level of 25(OH)D above 50 nmol/L or 20 ng/mL is deemed sufficient. Based off of thousands of studies documenting the negative health consequences of vitamin D levels between 20ng/mL and 30 ng/mL, some researchers believe that those recommendations are inadequate.\textsuperscript{35} The Endocrine Society’s guidelines reflect the needs of the much broader population. The Endocrine Society considers serum 25(OH)D levels below 20ng/mL to be deficient.\textsuperscript{36} Serum levels of 25(OH)D between 21ng/mL and 29ng/mL are considered insufficient by the Endocrine Society.\textsuperscript{36} Finally, serum levels of 25(OH)D 30ng/mL and above are considered sufficient.\textsuperscript{36} Additionally, the Endocrine Society also released daily intake guidelines for vitamin D. They recommend infants (0–1 years) to have 400-1000 IU per day, children (1–18 years) 600–1000 IU and adults (18 and older) to have 1500–2000 IU of vitamin D per day.\textsuperscript{36}
OBJECTIVES

The general consensus on vitamin D is that there is an increasing incidence of vitamin D insufficiency and deficiency globally. Very few foods are fortified with vitamin D. Oily fish such as salmon are known to be a good source of vitamin D. But little is known about the vitamin D content of other fish types.

The objective of this study is to analyze and elucidate specifically:

(1) the vitamin D content in sardines.

(2) the vitamin D content in mackerel.

Through this study it the vitamin D content of sardines and mackerel will be determined. Furthermore, through this study there will be a better understanding of how to better supplement one’s diet with naturally occurring vitamin D.
METHODS

Materials:

The sardines in water used came from 3 separate cans, sold by Chicken by the Sea, P.O. Box 308 Mt. Olive, NJ 07828. The mackerel in oil came from Vital Choice Wild Seafood & Organics, 2460 Salashan Loop, Ferndale, WA 98248l. The normal phase zorbax silica 5 micrometer HPLC column used was purchased from Agilent which is located at 5301 Stevens Creek Blvd, Santa Clara, CA 95051, United States. The reverse phase C-18 HPLC Vydac column used was purchased from Grace, which is located at 7500 Grace Drive, Columbia, MD, 21044, United States. The Agilent 1100 Series HPLC machine used purchased from Hewett Packard made in Germany but the location of the company is at 5301 Stevens Creek Blvd, Santa Clara, CA, 95051, United States.

Running a Vitamin D₃ Control Sample for HPLC:

100ng of Vitamin D₃ was used as my control sample and the stock solution was a concentration of 2ng/uL. 50 microliters of the stock solution was placed in a test tube and dried down with nitrogen. After drying, 140 microliters of normal phase running solution (0.08% isopropanol in hexane) was added to the test tube. From there, the 140 microliters was transferred into a HPLC running tube. The running tube was placed into the HPLC machine for 15 minutes at a flow rate of 1.5 milliliters per minute. The area under the curve for the vitamin D₃
peak, the vitamin D\textsubscript{3} absorption spectrum and time the peak appeared on the chromatogram were determined.

**Saponification:**

A whole fish was weighed and then placed in a mortar and pestle. The fish was then mashed for 10 minutes. It is from that ground up mass in which a one gram sample of the fish is taken. For each individual saponification reaction, one gram of the sample was placed in a 50mL sealable glass tube. From there 7mL of 200% ethanol was added to each glass tube along with 3 grams of potassium hydroxide (KOH) and 5mL of water. 100ng of vitamin D\textsubscript{2} was added as an internal standard. A sonicator was then used on medium setting before sealing the glass tube. Once sealed, paraffin wrap was used and placed on top of the cover as a precaution. The test tube was then placed on a shaker on medium speed for 8 hours. After that time has elapsed 15mL of hexane was added to the tube and shaken again for 30 minutes. After that time, the tube was let to settle for about 2 minutes. The top (hexane) layer was then removed and placed in a separatory flask. This step was repeated with another 15mL of hexane. After the second transfer, the separatory flask contained about 30mL of hexane. 100mL of water was added to the separatory flask. The separatory flask was then sealed and placed on the shaker for 30 minutes. After shaking it was settled in a vertical position for 60 minutes. Afterward, the bottom (water) layer was drained. This
washing process removed polar impurities. This step was repeated for a total of 3 times. At the end of the third wash, the hexane layer was placed in a glass tube again and dried down with nitrogen gas.

**Preparation for Normal Phase HPLC**

Once the extract was finished drying down 2 mL of normal phase running solvent (0.08% isopropanol in hexane) was added to the glass tube. The walls of the tube were washed with the 2mL to ensure higher recovery. Those two mL’s were pipetted out from that glass tube and transferred it to a smaller test tube. The contents of the smaller test tube were dried down with nitrogen gas. 140 microliters of running solvent was added to that test tube. The contents of that test tube were transferred to a HPLC running tube. At this point the HPLC was set to run each sample for 14 minutes with a flow rate of 1.5 milliliters per minute. HPLC solvent was collected between 10 and 14 minutes.

**Switching from Normal Phase HPLC to Reverse Phase HPLC**

Switching from normal phase HPLC to reverse phase HPLC consisted of going through two intermediate solutions with no column connected. Normal phase HPLC running solution is 0.08% isopropanol in hexane. Detach the column and a bypass connector is applied. The HPLC was then run with a
solution of 40% isopropanol in hexane for 25 minutes at 1.5mL per minute. After that time, the solution was switched to 100% MeOH for 25 minutes at 1.5mL per minute. After that time has elapsed, the solution was switched to the 20% MeOH in ACN. The HPLC was allowed to run for 20 minutes then the bypass connector was removed and the reverse phase column was applied.

**Preparation for Reverse Phase HPLC**

Each sample from the normal phase HPLC was taken to dry down under nitrogen gas. This was done to collect where the vitamin D would normally elute out (10-14 minutes, based off of the controls that were run). It was dissolved with 140 microliters of the reverse phase running solvent (20% MeOH in ACN) for preparation to be run in a C-18 reverse phase column. The 140 microliters was transferred to a HPLC glass tube. The HPLC machine was set to 1.0 milliliters per minute with a running time of 11 minutes. The area under the curve for the vitamin D$_2$ and vitamin D$_3$ peaks were determined.

**Construction of the standard curve**

The stock solution of vitamin D$_3$ had a concentration of 2ng/microliter. The standard curve included had the following increments: 25ng, 50ng, 100ng, 200ng, 400ng. 140 microliters of running solution (0.08% isopropanol in hexane)
was then pipetted into each of the test tubes. After dissolving and mixing, that 
140 microliters was pipetted into each of their own running tubes and placed in 
the HPLC. The HPLC was then set to run each of those 5 running tubes 
sequentially for a run time of 15 minutes at a flow rate of 1.5mL per minute using 
the normal phase running solution (0.08% isopropanol in hexane). The standard 
curve was generated by plotting area under the peak with concentration.
RESULTS

Figure 3. Control Chromatogram of 100ng of Vitamin D₂ on a Normal Phase HPLC detected at 265nm.

Figure 4. Ultraviolet Absorption Spectrum of the Vitamin D₂ Peak (12.07 minutes) in Figure 3.

Figure 3 and Figure 5 show the chromatograms of vitamin D₂ and vitamin D₃ from normal phase HPLC. The two peaks appear at the same time, this is expected because with normal phase HPLC it is difficult to discern between similar molecules. Vitamin D₂ and Vitamin D₃ are eluted out at 12.07 and 12.02 minutes, respectively.
Figure 5. Control Chromatogram of 100 ng of Vitamin D₃ on a Normal Phase HPLC column detected at 265nm.

Figure 4 and Figure 6 show the UV spectrum of vitamin D₂ and vitamin D₃ respectively. The UV spectrum is used to prove the presence of vitamin D since it has a characteristic appearance. Spectrums of vitamin D have a trough at 228nm and a peak at 265nm additionally the ratio of 2/3 should be present. Both Figures 4 and 6 have those characteristics.

Figure 6. Ultraviolet Absorption Spectrum of the Vitamin D₃ Peak (12.02 minutes) in Figure 5.
Figure 7. Chromatogram of a lipid extract of sardine packed in water on a HPLC column detected at 265nm.

Figure 7 is a chromatograph of one of the sardine samples under normal phase HPLC. The point of interest is the peak appearing at 12.00 minutes, based on the controls that were performed, that is where vitamin D would be expected to elute out. Figure 8 shows the spectrum of the peak at 12.00 minutes, and based on the spectra, that confirms where the vitamin D eluted out. During the normal phase HPLC the sample was collected, typically it was collected between 10-14 minutes. That solution was then dried down and saved for reverse phase HPLC.
Figure 8. Ultraviolet Absorption Spectrum of Vitamin D Peak (12.00 minutes) in Sardine Chromatograph from Figure 7.

Figure 9. Control Chromatogram of Vitamin D$_2$ on a Reverse Phase HPLC
Figure 9 and Figure 11, respectively show the chromatographs for vitamin D$_2$ and vitamin D$_3$. The important notion here is that vitamin D$_2$ eluted out earlier than vitamin D$_3$. In Figure 9, the vitamin D$_2$ peak eluted at 8.49 minutes. In Figure 11, the vitamin D$_3$ peak eluted at 9.27 minutes. This was an important because with reverse phase it was possible to distinguish between vitamin D$_2$ content and vitamin D$_3$ content. Figure 10 and Figure 12 show the ultraviolet absorption spectrum for vitamin D$_2$ and vitamin D$_3$ respectively.
Figure 11. Control Chromatogram of Vitamin D₃ on a Reverse Phase HPLC

Figure 12. Ultraviolet Absorption Spectrum of Vitamin D₃ Peak in Figure 11.
Figure 13. Chromatogram of Vitamin D₂ and Vitamin D₃ on RHPLC

Figure 13 shows the combined control, which had both vitamin D₂ and vitamin D₃. It is clear that they appear as separate peaks, which made distinguishing between vitamin D₂ and vitamin D₃ possible. Figure 14 and Figure 15 show the ultraviolet absorption spectrum for vitamin D₂ and vitamin D₃, respectively. Both of the spectrums look identical. Both have a peak at 265nm and a tough a 228nm and a 2/3 ratio.
Figure 14. Vitamin D$_2$ Ultraviolet Absorption Spectrum from the Combined Control in the Figure 13

Figure 15. Vitamin D$_3$ Ultraviolet Absorption Spectrum from the Combined Control from Figure 13
Figure 16. Lipid extract packed in water sample Reverse Phase High Performance Liquid Chromatography

Figure 16 is a chromatograph of a sardine lipid extract on a reverse phase HPLC. Just as observed in the control in Figure 13, both the vitamin D$_2$ and vitamin D$_3$ peaks were visible.

Figure 17. Ultraviolet Absorption Spectrum of Peak at 8.48 Minutes from Figure 16.
Figure 18. Ultraviolet Absorption Spectrum of Peak at 9.19 Minutes from Figure 16.

Figure 17 and Figure 18 are the ultraviolet spectra for vitamin D$_2$ and vitamin D$_3$. These spectra confirm that those two peaks are vitamin D. And based on the controls that were performed as seen in Figure 9, Figure 11, and Figure 13, the peak at 8.5 minutes is vitamin D$_2$ and the peak at 9.2 minutes is vitamin D$_3$.

Figure 19. Chromatogram of a lipid extract of mackerel packed in oil on a HPLC column detected at 265nm.
Figure 19 shows a chromatogram of a lipid extract of a mackerel packed in oil. The peak of interest is the peak at 11.50 minutes. Based on the controls that were performed, that is where vitamin D would be expected. Based on Figure 20, that confirmed that the peak at 11.50 minutes was vitamin D.

Figure 20. Ultraviolet Absorption Spectrum of the Vitamin D Peak (11.50 minutes) in Figure 19.

Figure 21. Chromatogram of a lipid extract of mackerel packed in oil on a reverse phase HPLC column detected at 265nm
Figure 21 is a chromatogram of a lipid extract of mackerel packed in oil. The two peaks of interests are the peak at 7.5 minutes and the peak at 8.21 minutes. Based on the controls, those are the two areas that would be expected to have vitamin D$_2$ and vitamin D$_3$, respectively. Figures 22 and 23 show the ultraviolet absorption spectrum of those two peaks. Those spectra confirm that they are vitamin D. It is known that D$_2$ is eluted first and then vitamin D$_3$.

Figure 22. Ultraviolet Absorption Spectrum of the Vitamin D$_2$ Peak (7.6 minutes) in Figure 21

Figure 23. Ultraviolet Absorption Spectrum of the Vitamin D$_3$ Peak (8.2 minutes) in Figure 21
Figure 24. Standard Curve Vitamin D\textsubscript{3} on a normal phase HPLC

Figure 24 is a standard curve of vitamin D\textsubscript{3} on a normal phase HPLC. The purpose of the standard curve is to be able to determine the amount of vitamin D in nanograms for a given area under the peak on the chromatogram. To construct this standard curve, amounts of the following: 25ng, 50ng, 100ng, 200ng, and 400ng, were analyzed in on normal phase HPLC. So for example, if the area under the peak for a vitamin D\textsubscript{3} was 100, then the amount would be 75ng.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fish 1</th>
<th>Fish 2</th>
<th>Fish 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>234.4</td>
<td>266.1</td>
<td>230.5</td>
</tr>
<tr>
<td>Sample 2</td>
<td>235.8</td>
<td>302.9</td>
<td>233.1</td>
</tr>
<tr>
<td>Sample 3</td>
<td>263.8</td>
<td>262.5</td>
<td>238.1</td>
</tr>
<tr>
<td>Sample 4</td>
<td>241.6</td>
<td>491.6</td>
<td>395.1</td>
</tr>
<tr>
<td>Sample 5</td>
<td>250.3</td>
<td>283.4</td>
<td>298.9</td>
</tr>
</tbody>
</table>

Table 1. Sardine Data HPLC – Area Under The Peaks
Table 1 shows the raw data for area under the peaks for the vitamin D for
sardines on a normal phase HPLC. Five samples were processed from each
sardine. These values are much higher than the values from the reverse phase
because these raw data encompass the vitamin D$_2$ and vitamin D$_3$ within the
peak.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fish 1 D$_2$</th>
<th>Fish 1 D$_3$</th>
<th>Fish 2 D$_2$</th>
<th>Fish 2 D$_3$</th>
<th>Fish 3 D$_2$</th>
<th>Fish 3 D$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>91.7</td>
<td>81.6</td>
<td>106.1</td>
<td>107.5</td>
<td>106.0</td>
<td>104.4</td>
</tr>
<tr>
<td>Sample 2</td>
<td>91.3</td>
<td>124.8</td>
<td>113.7</td>
<td>138.7</td>
<td>90.7</td>
<td>98.6</td>
</tr>
<tr>
<td>Sample 3</td>
<td>112.9</td>
<td>83.8</td>
<td>113.3</td>
<td>134.4</td>
<td>97.1</td>
<td>95.7</td>
</tr>
<tr>
<td>Sample 4</td>
<td>114.4</td>
<td>91.6</td>
<td>111.1</td>
<td>133.0</td>
<td>152.8</td>
<td>169.1</td>
</tr>
<tr>
<td>Sample 5</td>
<td>104.2</td>
<td>122.7</td>
<td>100.6</td>
<td>120.3</td>
<td>107.7</td>
<td>108.3</td>
</tr>
</tbody>
</table>

Table 2 shows the data of fractions collected from the straight phase
HPLC. Each sardine lipid extract had 100ng of vitamin D$_2$ as an internal standard.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fish 1</th>
<th>Fish 2</th>
<th>Fish 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>59.4ng</td>
<td>78.3ng</td>
<td>75.9ng</td>
</tr>
<tr>
<td>Sample 2</td>
<td>90.7ng</td>
<td>100.9ng</td>
<td>71.8ng</td>
</tr>
<tr>
<td>Sample 3</td>
<td>60.9ng</td>
<td>97.8ng</td>
<td>69.6ng</td>
</tr>
<tr>
<td>Sample 4</td>
<td>61.0ng</td>
<td>96.7ng</td>
<td>123.1ng</td>
</tr>
<tr>
<td>Sample 5</td>
<td>89.3ng</td>
<td>87.5ng</td>
<td>78.5ng</td>
</tr>
</tbody>
</table>

Table 3 shows the Vitamin D$_3$ content per Sample – Sardines.

Table 3 shows the Vitamin D$_3$ content in nanograms per each one gram
sample of sardine. This was calculated by taking the area under the curve and
multiplying that by 0.4 and multiplying that by 1.4. The conversion factor of 0.4 is
a previously established conversion factor for vitamin D. The factor of 1.4 is
based off of the amount of running fluid that was placed in the HPLC glass
tubing. Based off of the internal standard of vitamin D$_2$, I had a 70% recovery of
vitamin D. So the values would then be increased by 30%. Table 3 reflects that increase. Ultimately, that would result in the amount of vitamin D₃ per gram of sardine. Table 4 shows the average vitamin D₃ content per gram of fish with their standard deviations.

<table>
<thead>
<tr>
<th></th>
<th>Fish 1</th>
<th>Fish 2</th>
<th>Fish 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Vitamin D₃</td>
<td>72.3 ng</td>
<td>92.2 ng</td>
<td>83.8 ng</td>
</tr>
<tr>
<td>per Gram of Fish</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>16.2</td>
<td>9.3</td>
<td>17.1</td>
</tr>
</tbody>
</table>

Table 4. Average Vitamin D Content per Gram of Sardine Fish

<table>
<thead>
<tr>
<th>Overall Average Vitamin D₃ per gram of Sardine</th>
<th>82.7 ng</th>
</tr>
</thead>
</table>

Table 5. Overall Average of Vitamin D₃ per gram of Sardine

Table 5 shows the overall average vitamin D₃ per gram of sardine. This data was calculated by averaging the each of the sample means. Table 6 provides information about the weight of each sardine fish, and the amount of IU’s of vitamin D₃ present in each sardine fish. The average for sardines is 82.7 ng of vitamin D₃ per gram of fish. An average serving size of sardines 3.5 ounces which is the same as 100 grams. So an average serving of sardines has 8270 ng of vitamin D, which is equal to 330.8 IU's.

<table>
<thead>
<tr>
<th></th>
<th>Fish 1</th>
<th>Fish 2</th>
<th>Fish 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish Weight (g)</td>
<td>18.0</td>
<td>25.85</td>
<td>17.93</td>
</tr>
<tr>
<td>IU per fish</td>
<td>40.69</td>
<td>70.30</td>
<td>46.40</td>
</tr>
</tbody>
</table>

Table 6. IU per Sardine Fish
Table 7. Normal Phase HPLC – Mackerel Data – Area Under the Peaks

<table>
<thead>
<tr>
<th>Mackerel 1</th>
<th>Mackerel 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>360.9</td>
</tr>
<tr>
<td>Sample 2</td>
<td>476.9</td>
</tr>
<tr>
<td>Sample 3</td>
<td>319.1</td>
</tr>
<tr>
<td>Sample 4</td>
<td>295.9</td>
</tr>
<tr>
<td>Sample 5</td>
<td>Not Available</td>
</tr>
</tbody>
</table>

Table 8. Reverse Phase HPLC – Mackerel Data – Area Under the Peaks

<table>
<thead>
<tr>
<th>Mackerel 1 D&lt;sub&gt;2&lt;/sub&gt; Peak</th>
<th>Mackerel 1 D&lt;sub&gt;3&lt;/sub&gt; Peak</th>
<th>Mackerel 2 D&lt;sub&gt;2&lt;/sub&gt; Peak</th>
<th>Mackerel 2 D&lt;sub&gt;3&lt;/sub&gt; Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>None</td>
<td>None</td>
<td>99.3</td>
</tr>
<tr>
<td>Sample 2</td>
<td>21.4</td>
<td>None</td>
<td>88.6</td>
</tr>
<tr>
<td>Sample 3</td>
<td>None</td>
<td>None</td>
<td>95.5</td>
</tr>
<tr>
<td>Sample 4</td>
<td>None</td>
<td>None</td>
<td>74.2</td>
</tr>
<tr>
<td>Sample 5</td>
<td>Not Available</td>
<td>Not Available</td>
<td>104.5</td>
</tr>
</tbody>
</table>

Table 9. Vitamin D<sub>3</sub> per Gram of Mackerel Sample.

| Sample 1 | None Recovered | 23.7 ng |
| Sample 2 | None Recovered | 15.6 ng |
| Sample 3 | None Recovered | 19.0 ng |
| Sample 4 | None Recovered | 22.7 ng |
| Sample 5 | Not Available  | 21.2 ng |

Table 10. Average Vitamin D<sub>3</sub> content per gram of mackerel

<table>
<thead>
<tr>
<th>Average Vitamin D&lt;sub&gt;3&lt;/sub&gt; Content per Gram of Mackerel</th>
<th>20.4 ng</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Deviation</td>
<td>3.23</td>
</tr>
</tbody>
</table>

Table 7 shows the raw data from the HPLC machine. Mackerel # 1 sample 5, was destroyed during the experiment due to high pressure in the running column. Since it was destroyed in the process during normal phase HPLC, there is consequently no data for that sample with reverse phase HPLC.

Table 8 shows the raw data after reverse phase HPLC. There was no vitamin D<sub>3</sub> recovered at all for Mackerel # 1. Mackerel # 2 had consistent results.
for vitamin D₃. Table 9 shows the vitamin D₃ per gram of mackerel sample.

Based off of the internal standard of vitamin D₂, I had 70% recovery. The additional 30% is factored into Table 9.

An average serving size of mackerel is 3.5 ounces, which is the same as 100 grams. So therefore there are 2040 ng of Vitamin D₃ in a standard serving of mackerel. 2040 ng of vitamin D is equal to 81.6 IU's of vitamin D.
DISCUSSION

A 3.5 ounce can of tuna has roughly 230 IU of vitamin D$_3$. And fresh wild salmon has roughly 600-1000 IU per 3.5 ounces. 8 ounces of orange juice or milk both have about 100 IU of vitamin D$_3$. Cereal has about 100 IU per serving size. Based on the results from this experiment a standard serving, which is 3.5 ounces (3 fish), of mackerel in oil has about 81.6 IU’s of vitamin D$_3$, and a standard serving of sardines (3.5 ounces) has about 330.8 IU’s of vitamin D$_3$. One single mackerel fish has about 27.2 IU’s of vitamin D. One single sardine fish has about 66.2 IU’s of vitamin D. So in comparison to the fortified juices and cereal the canned sardines have more vitamin D content overall. But in comparison to salmon, the sardines only provide roughly 25% to 50% of the vitamin D compared to wild caught salmon. In comparison to farmed salmon, the vitamin D content in sardine is roughly the same. In comparison to some of the fortified foods such as milk and even the non-fortified foods such as wild salmon; mackerel does not seem to have an overall high vitamin D$_3$ content. But for people who are vitamin D deficient or insufficient, supplementing mackerel or sardine in one’s diet can be one way to increase one’s serum 25(OH)D levels.

Some limitations of this study consist of the fact that the fish only came from two companies. The sardines only came from Chicken of the Sea and the Mackerel only came from Vital Choice. Additionally, another limitation to the study is that these experiments only dealt with canned fish and not fresh or frozen fish. Furthermore, another limitation is that the mackerel fish were packed
in oil. Since vitamin D is lipid soluble, there is a chance that some of the vitamin D from the mackerel may have leached into the oil.

One minor limitation and something that went awry for the mackerel is that, only two fish were (10 samples) were run instead of 15 like with sardines. 10 samples should have been sufficient but one sample was destroyed in the beginning to do a pressure problem in the normal phase column. And for the first mackerel fish run (mackerel # 1), there was no recovery of vitamin D$_3$ and almost no recovery of vitamin D$_2$ for reverse phase. Ultimately this resulted in only 5 viable complete samples of mackerel. One theory for this is that during the saponification process, some of the sample spilled out. One other probable theory is that there was a problem with the extraction step.

One other minor limitation to this study is that the sardines and mackerel used in the study came from the same area. All the sardines came from Thailand and all the mackerel came from Portugal. Perhaps depending on the environment, mackerel or sardine in other geographic areas may have more or less vitamin D than the samples studied in this experiment. Additionally, the time of year that the fish were harvested can impact the vitamin D content in fish. It is known that in wild salmon, there is a seasonal effect on vitamin D. With wild salmon, vitamin D content is high when the fish are caught during the summer.

Some areas of future research include investigating the vitamin D content in deep sea fish species such as the lanternfish or anglerfish which exist at depths where there is no visible light. Those two fish species live in total
darkness, and therefore it would be interesting to see if those fish can create vitamin D and by what biological pathway is it done. Another area of future research is to investigate other common fish and aquatic species for their vitamin D content. It would be interesting to note the difference in metabolism and vitamin D content between crustaceans such as lobsters, crabs, and shrimp versus common fish such as red snapper, and blue fish.
REFERENCES


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CURRICULUM VITAE

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Year of Birth: 1991

Education: Boston University
Bachelor of Science in Human Physiology, May 2013

Boston University School of Medicine, Boston, MA
Candidate for Master of Science of Medical Science, May, 2015

Research Experience

09/14 to Present
Boston University Medical Center

• Boston University
• Boston, MA
• Research assistant in Dr. Michael Holick’s vitamin D lab. Lab work focused on extraction of vitamin D from fish.

09/10 to 12/10
Boston University Medical Center

• Boston University
• Boston, MA
• Research Assistant in Dr. Zhdanova’s neuroscience lab. The focused on the effects of pharmacologic agents on the circadian rhythm of zebra fish. I assisted in the administration of the pharmacologic agents, maintenance of the zebrafish, conducted western blots, and conducted dissections.
Medically Relevant Experiences

06/12 to 09/13  Boston University EMS
• Boston, MA
• Emergency Medical Technician
• Responsible for providing first-aid and pre-hospital interventions to patients in various facilities at Boston University.

03/12 to 05/13  Medical Career Exploration Program
• Brigham and Women's Hospital
• Boston, MA
• Emergency Department Volunteer & Student Ambassador
• Responsible for transporting patients and specimens to and from various departments in the hospital. As well as assist with accommodating and helping patients in the Emergency Department.

10/10 to 05/11  Health LEADS
• Dimock Center
• Boston, MA
• Patient Advocate
• Assisted the clinical staff in an effort to improve patients' healthcare via non-medical but yet health essential factors of life.

Volunteer Work

10/10 to 04/12  Global Water Brigades
• Tegucigalpa, Honduras
• Treasurer
• Led several water brigades to Honduras, and helped to build and design water purification systems for rural Honduran communities.

09/10 to 05/11  Student Food Rescue
• Boston University
• Boston, MA
• Volunteer Driver
• Volunteered in picking up food from various restaurants in Boston and delivered it to several homeless shelters around Boston.