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Detection of PAHs in Commercial and Wild Caught Fish Oil using Scanning Fluorescence Spectroscopy

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By

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Abstract:

The DeepWater Horizon (DWH) oil spill contaminated a large area in the Gulf of Mexico in summer 2010. It is likely that many aquatic species in the Gulf were affected by the spill. Crude oil contains polycyclic aromatic hydrocarbon (PAHs). Some PAHs are carcinogenic to fish and humans. The purpose of this project was to develop menhaden fish oil as a biomonitoring tool for crude oil contaminants such as PAHs using scanning fluorescence spectroscopy (SFS). Menhaden (Genus *Brevoortia*) is one of the most ecologically and economically important marine fish species along the Atlantic and Gulf coast; however, it is also one of the least studied. Menhaden are a filtering feeding fish and very important to aquatic ecosystems as the principle prey species for higher trophic level organisms, such as bluefish and tuna. Menhaden are also important to the bait and reduction industries, including the production of omega-3-fatty acid fish oil tablet supplement. It was hypothesized that their body oil would accumulate crude oil contaminants such as PAHs. Fish oil was obtained by separating fish muscle and skin from internal organs.

Crude oil contamination has commonly been tracked by measuring PAHs in fish bile and or tissues using fluorescence. Vitamins are fluorescent compounds, and it is possible that these vitamins are confounding factors when trying to measure PAHs in biological samples using fluorescence. In order to evaluate this, SFS was used to detect PAHs and vitamin standards alone and when combined with fish oil. Fish oil was obtained from several "over the counter" brands (such as Nature's Bounty), a prescription brand (Lovaza), a commercial product (menhaden oil from DayBrook Industries) and wild menhaden collected from the Delaware Bay, NJ, James River, VA, Vermillion Bay, LA and Barataria Bay, LA in 2010 and/or 2011. Barataria Bay received heavy oiling from the DWH spill from April to August 2010. Vitamins and PAH

standards were bought commercially. The fish oils and standards were analyzed using a SpectraMax M5 which provided a 2D scan and a Fluorolog 3 which provided a 3D scan.

Results indicated the presence of both PAH-like and vitamin-like compounds in fish oils. 2D spectra showed peaks in wild fish samples at Em350/Ex280, Em350/Ex290, Em450/Ex320 and Em450/Ex350. Comparisons to standards indicated that the peak at Em350/Ex290 could be vitamin E, the one at Em350/Ex280 could be albumin and/or naphthol, the one at Em450/Ex320 could be vitamin A and the one at EM450/Ex350 could be high MW PAHs. The presence of most of these compounds was supported by 3D spectra, which were better able to distinguish between standards than 2D spectra. Only vitamin A in wild fish oils was not confirmed by 3D spectra. Commercial fish oils contained mostly vitamin E. Lovaza (LV) appeared to contain vitamin A and Nature's Bounty (NB) appeared to contain low MW PAH-like compounds. Spectra were also generated for DWH crude oil and for bile from DWH exposed killifish. 2D and 3D spectra for these samples were highly similar to several wild fish oil samples including three NJ collections and the one from Barataria Bay, LA collected in spring 2011. Overall, results indicated that vitamins and protein may be confounding factors when detecting naphthol-like PAHs in fish oil using fluorescence technologies. Some wild fish oils appeared to contain PAH-like compounds while others did not, indicating that menhaden fish oil is a promising biomonitoring tool. However, PAHs accumulated from crude oil could not be distinguished from those acquired from urbanization.

Introduction:

Crude oil contamination is an ongoing environmental issue, and the release of crude oil from the British Petroleum's (BP) DeepWater Horizon (DWH) Oil Rig has caused a major concern for the Gulf of Mexico's fisheries, in particular the impact of petroleum on wildlife. The BP's Mississippi Canyon 252 ruptured pipeline began on April 20th, 2010 and continued for 87 days until the well was capped on August 3rd, 2010 (Yu et al, 2011). The DWH incident has contributed a total of 699,700 metric tons of crude oil spill (McNutt et al, 2012). The rig explosion killed 11 workers and leaked approximately 500-800 thousand gallons per day (Yu et al, 2011).

Crude oil is a complex mixture that has been broken down into four major chemical groups including alkanes, cycloalkanes, aromatic hydrocarbons, and asphaltic (Robbins and Hsu, 1996).The DWH catastrophe has caused a major concern for wildlife because water pollution can cause health effects and threaten aquatic life including fish. Oil spills have been shown to cause strong toxic effects on exposed organisms by diverting their energy from growth and reproduction to sustaining their lives (Heintz 2007). Research has shown that toxicity from crude oil can affect the expression of some immune-related genes of the Atlantic cod fish (Perez-Casanova et al, 2012). The toxicity of oil can act like narcotics or anesthetics; such that, non-lethal doses can slow down the organism's ability to respond to their ecosystem whether it includes catching a prey or running from a predator (Yu et al, 2011).

Major sources for polycyclic aromatic hydrocarbons (PAHs) include crude oil spillage and incomplete combustion of coal, oil, gasoline and trees (Walker et al, 2006). These pollutants can enter the marine ecosystem and remain suspended in water in the form of oil droplets (Murawski et al, 2014). Once in this form, they can enter food chains and travel long distances

within migrating fish. The organism can take up PAHs and store them in adipose tissue until metabolism and excretion can occur (Yu et al, 2011). Metabolism of PAHs can occur in all tissues. Through metabolism PAHs can be transformed into carcinogenic metabolites that have the ability to interact with DNA and cause mutations that lead to cancer (Neff et al, 1979). The United States has one of the world's highest incidences of environmental cancer. For instance, 6% of cancer deaths are caused by environmental pollutants with nearly 11,000 deaths a year (Yu et al, 2011), and an estimated 1,500 people a day died of cancer in the U.S in 2010 (American Cancer Society, 2010). Cancer is the alteration of the genetic material that leads to production of abnormal proteins and to continuous cell division indefinitely. The increase in cancer incidence and mortality has shown to be the result of the increase in pollution being released into the environment through human activities (Yu et al, 2011). PAHs in the environment may have a major role in cancer deaths due to the heavy use of fossil fuels by our society. Therefore, research on environmental toxics such as PAHs has to become a major priority.

PAHs are often measured in aquatic organisms as an indicator of crude oil exposure. PAHs have been routinely used for biomonitoring crude oil spills (Krahn et al, 1993, Jewetta et al, 2002, Barsiene et al, 2006, Jung et al, 2011) and oil combustion related contamination (Aas et al, 2000, Jonsson et al, 2004, Trisciani et al, 2011) due to their simplicity of detection by fluorescent techniques (Ariese et al, 1993, Lin et al, 1996, Kim et al, 2010). The aim of this project was to determine if the pelagic fish, menhaden, are a suitable organism for examining oil spill impacts using their body oil to detect PAHs from crude oil exposure. Due to menhaden's oily nature, it was hypothesized that menhaden would accumulate and retain PAHs. Oil dissolves into oil, and PAHs are hydrophobic; therefore, fish oil will contain crude oil. Sampling of about

8,000 seafood specimen following the DWH incident for seafood safety has revealed low concentrations of PAHs in muscle (Ylitalo et al, 2012).

Aromatic compounds are luminescent compounds that can emit spontaneous radiation from an electronically excited state or a vibrational excited state not in thermal equilibrium (Braslavsky et al, 2007). The mode of excitation is absorption of one or more photons which brings the absorbing species into an electronic excited state (Valeur et al, 2013). In particular, photon absorption consists of energy passage from lowest energy (ground state) to higher energy (excited state). Fluorescence techniques are very sensitive methods and the ability to detect emitted light provides a very simple method for detecting fluorescent contaminants, particularly PAHs. Techniques for measuring PAHs in biological samples have included fixed wavelength fluorescence (FF), scanning fluorescence spectroscopy (SFS), high performance liquid chromatography with fluorescence detection (HPLC-F) and gas chromatography with mass spectrometry detection (GCMS). FF and SFS have easy and rapid methods of sample preparation that are suitable for biomonitoring (Lin et al, 1996, Aas et al, 2000, Jonsson et al, 2004). Different PAHs have been distinguished from one another because their aromatic rings can be excited by a specific wavelength, and this is followed by fluorescent light emitted at a longer, specific wavelength (Picture 1). The specificity for excitation and emission wavelengths has allowed detection of particular types of PAHs.

Menhaden (Family *Clupeidae*, Genus *Brevoortia*) is a marine teleost fish that is especially important to the commercial fisheries of the Gulf and Atlantic coasts; such that it is considered one of the most economically important species in North America (Franklin, 2007). Research has revealed four species of menhaden that live in North America: Brevoortia *tyrannus*, B. *smithi*, B. *patronus* and B. *gunteri* (Ahrenholtz, 1991). Menhaden is an oily, prey species used

in the bait and reduction industries as well as for making fish oil (McMillin et al, 1992). Menhaden is heavily fished for bait, pet food and essential fatty acid consumption. Menhaden feed largely at the surface or within several meters of the surface; therefore, they are likely to come into direct contact with crude oil spills or dispersed oil droplets. Due to their oily nature, menhaden may accumulate petroleum oil contaminants and potentially spread it to predatory fish such as: silver perch, blue fish, white bass, tuna, mackerel, and salmon thereby affecting marine ecosystems (Del Rio et al, 2010) as well as people that consume omega-3-fatty acids from supplemental fish oil tablets. Therefore, it may be possible that by consuming fish oil, humans may be exposed to oil contaminates such as PAHs. Some PAHs are known carcinogens.

Cardiovascular disease (CVD) is one of the most lethal human diseases and is very common in the United States (Go et al, 2012). CVD accounted for 40.7% of mortality in 1950, and unfortunately, it has only dropped to 20% by 2005, ranking it the number 1 cause of death with cancer ranking number 2 (Yu et al, 2011). It has been a popular topic for the past few years and prevention is essential. An effective way to help prevent CVD is by taking fish oil, which is rich in omega-3 fatty acids (Demiroglu et al, 1991), and appears to lower cholesterol (Mozaffarian and Wu, 2012). Population studies have indicated that fish intake and consumption of omega-3 fatty acids may result in a healthier lifestyle (Nordoy, 2001). In addition, epidemiological and clinical trials have demonstrated that omega-3 supplements can reduce incidences of CVD and reduce cardiac occurrences as well as decrease the progression of atherosclerosis in coronary patients (Kris-Eherton et al, 2002). Greek researchers reported that fish oil supplementation (10 grams/day) reduces the number of heart attacks by 41% in men suffering from angina, which is the most common symptom of CVD (http://www.mercola.com/beef/omega3_oil). Therefore, CVD research has shown that taking fish

oil supplements should be part of a healthy lifestyle. However, accumulation of PAHs by fish used to make these supplements may lead to transfer of these contaminants to humans. A sensitive technique for measuring PAHs in fish oil samples is needed in order to investigate any risks associated with consumption of fish oil supplements.

For this study, menhaden were collected from the Barataria Bay and Vermillion Bay, LA, Delaware Bay, NJ and the James River, VA. The Barataria Bay was highly impacted by the DWH incident because it received heavy oiling (Ramsey et al, 2011). The Delaware Bay and James River were not exposed to the DWH oil spill but are likely exposed to other sources of PAHs associated with urbanization. By comparing these two groups of fish, it may be possible to distinguish PAHs from crude oil spill versus PAHs from anthropogenic sources. The Vermillion Bay, LA was chosen as a possible negative control as it received less oiling from the DWH oil spill than Barataria Bay, LA. The James River, VA was chosen because two species of menhaden, *B. tyrannus* (Atlantic menhaden) and *B. patronus* (gulf menhaden) are found there. *B. tyrannus* migrates north past Delaware Bay, while *B. patronus* migrates south toward Florida and is one of the two species found in the Gulf of Mexico, the other is *B. smithi*. The data presented represents fish collected in fall of 2010 and summer 2011.

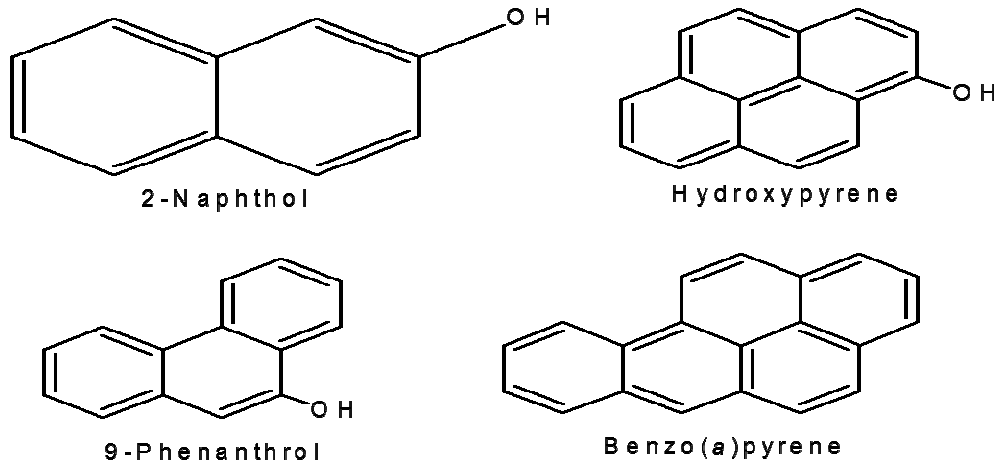
The quantities of particular PAHs have been assigned to distinguish between petrogenic and pyrogenic sources (Krahn et al, 1993). Pyrogenic sources of PAHs are associated with combustion of oil-based products such as gasoline, and petrogenic sources of PAHs are associated with petroleum releases such as the DWH oil spill. High levels of naphthalene and phenanthrene have been associated with crude oil exposure contamination, and high levels of pyrene and benzo (a) pyrene PAHs have been associated with oil combustion contamination (Aas et al, 2000 and Trisciani et al, 2011). Menhaden from the Delaware Bay and James River,

which were not exposed to a recent, major oil spill event, may be exposed to PAHs from pyrogenic sources. Comparing PAHs in their fish oil to those from LA may allow the detection of a DWH crude oil "fingerprint" distinguishable from a pyrogenic background.

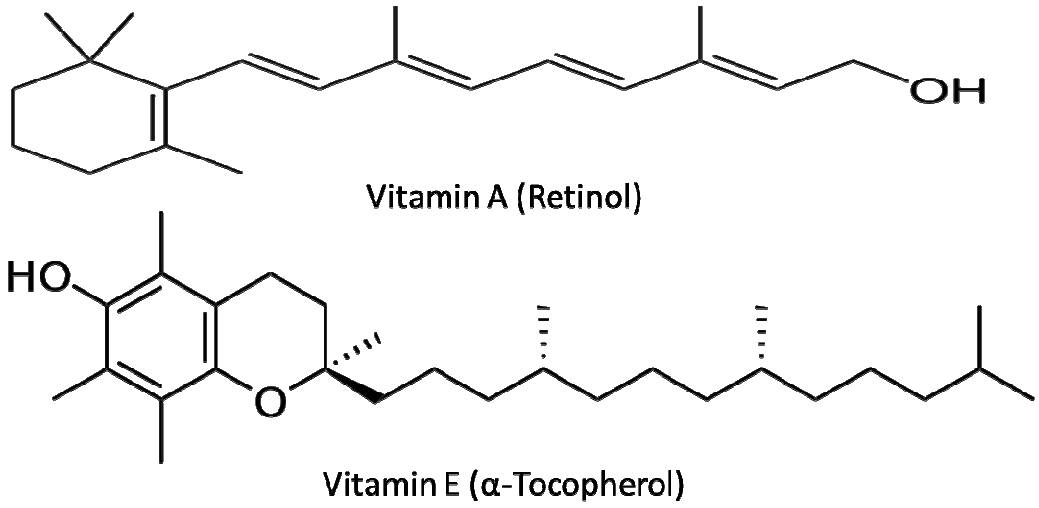
Research on menhaden has found that they are first order consumers of phytoplankton (Durbin et al, 1998). Phytoplankton has been found to contain high levels of vitamins A and E (Dunlap et al, 2002 and Vincent, 2014). Vitamin E is not only a natural component of fish oil (Aidos et al, 2002 and McGuire et al, 1997), fish oil manufacturers have added small amounts of vitamin E to prevent spoilage, and it has been combined with other vitamins including vitamins A, B1, B2, B3, C and D (http://www.webmd.com/vitamins-supplements). Since vitamins A and E contain an aromatic ring, their fluorescence under test conditions may have made them confounding factors when trying to measure PAHs using fluorescence (Picture 2). Many researchers have used fluorescence to biomonitor PAHs in fish, particularly using bile and tissue (Baussant et al, 2000 and Barsiene et al, 2006). Researchers have frequently used fixed wavelength settings to detect certain PAHs extracted in 48% ethanol (EtOH) (Kreitsberg, et al, 2010); however, other biological fluorescent compounds like proteins and both vitamins A and E may be detected using these same wavelengths. This could have resulted in an overestimation of PAH exposure by these researchers. This project investigated whether or not proteins and vitamins A and E could be confounding factors when measuring PAHs in fish oil using fluorescence technologies.

The aim of the project was to determine whether menhaden fish oil is a suitable matrix for biomonitoring PAHs. Fluorescence detection was chosen because of the easy of sample preparation, its sensitivity for fluorescent compounds, and its ability to distinguish between different types of PAHs. Development of fish oil as a biomonitoring tool involved determining

what solvent to use for extraction, the extent to which fluorescent compounds in the fish oil could be recovered from the matrix, and what wavelengths to scan in order to detect different types of PAH compounds. Fish oil samples included both commercial fish oil supplements and fish oil from wild menhaden caught in NJ and LA. PAHs in commercial fish oil could pose a problem for humans consuming these supplements as part of a heart healthy program. Wild fish oil may allow biomonitoring of crude oil spills and urban sources of PAHs as well as the ability to distinguish between the two.



Picture 1. Demonstrate the molecular structures of PAHs standards that were analyzed using a Spectramax M5 and a Fluorolog 3. Note the number of rings for each PAHs characterizes whether it is a low MW PAH versus a high MW PAH. The number of rings can also characterize their level of toxicity. For example, Benzo (a) pyrene contains 5 rings which makes it a high MW PAH and also harder to metabolite which can lead to genotoxicity and mutagenesis. All PAHs drawings were done in ACDlabs ChemSketch on June 4, 2012.



Picture 2. Demonstrates the molecular structure of Vitamin A and Vitamin E. Note that both vitamins have a ring which allows fluorescence. Vitamin A and E are found in fish oil; therefore, it was noted that both vitamins may be detected instead of PAHs when using SFS.
http://en.wikipedia.org/wiki/File:All-trans-Retinol.svg
http://en.wikipedia.org/wiki/File:Tocopherol,_alpha-.svg

Materials/Methods

Collection Sites

Menhaden were collected in fall 2010 from three locations (Figure 1). One was in Barataria Bay, LA which is near Grand Isle. This site received heavy oiling from the Deep Water

Horizon (DWH) oil spill. The second site was offshore at the mouth of Delaware Bay, NJ. This area has not had a recent, major oil spill. The Delaware Bay borders major metropolitan areas such as Philadelphia, PA and Trenton, NJ. NJ fish oils were identified based on the ship from which they were caught, such as Enterprise or Mount Vernon. The third site was the James River, VA. The James River, VA was chosen because two species of menhaden, *B. tyrannus* (Atlantic menhaden) and *B. patronus* (gulf menhaden) are found there. Menhaden were also collected in summer 2011 from Vermillion Bay, LA as well as Barataria Bay, LA. Vermillion Bay is west of Barataria Bay and was not directly impacted by the DWH oil spill. Menhaden were collected by staff of NJ Fish and Wildlife, LA Wildlife and Fisheries or Virginia Institute of Marine Sciences.

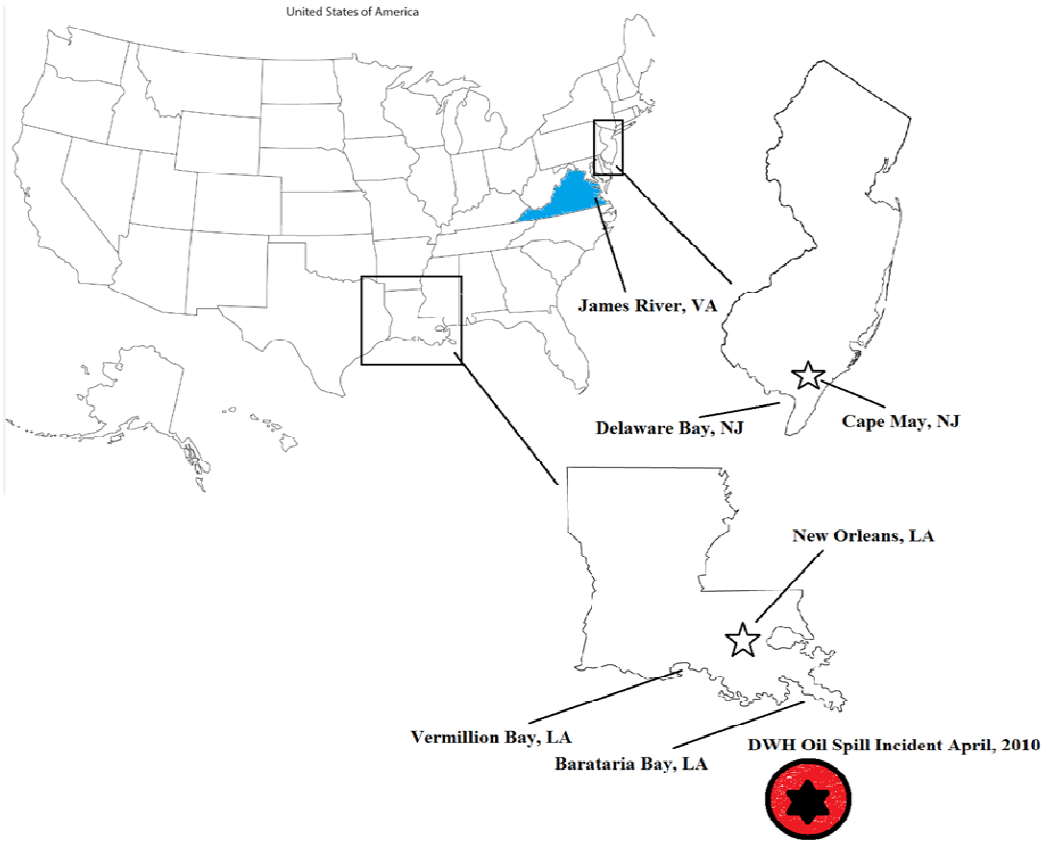


Figure 1. Locations where menhaden were collected, Barataria Bay, LA and Delaware Bay, NJ. Atlantic menhaden were collected from Cape May, NJ during the Fall of 2010. Gulf menhaden were collected from Barataria Bay near Grand Isle, LA and in the James River, VA during Fall of 2010. Menhaden were also collected from Vermillion Bay, LA and Barataria Bay, LA in the summer of 2011. Note the "X" represents the direction where the DHW incident occur in April 2010.

Fish Oil

Fish oil was obtained from commercial sources, "over the counter", or made in the laboratory from wild menhaden fish caught from NJ, LA or VA. Commercial fish oils were: Nature's Bounty, lot number 371123-0; GNC, lot number 4114HK8886; Nature Made lot number 1074121; Spring Valley fish oil, provided by Alexandria Garcia, Seton Hall University; and Lovaza, a prescription brand provided by Dr. Allan D Blake, Seton Hall University. DayBrook fish oil was a commercial product provided from DayBrook industries. It was prepared May 28, 2009, which was one year prior to the April 2010 DWH spill. Fish oil from wild caught menhaden included Barataria Bay, LA (BBLA) 2010 and 2011, Vermillion Bay, LA (VBLA) 2011, James River, VA (JRVA) 2010 and NJ seining boats- Enterprise (EPNJ), Sea Huntress (SHNJ) and Mount Vernon (MVNJ). For EPNJ and MVNJ, there were two separate collection dates designated by adding 1 or 2 to the acronym. A separate sample of fish oil was prepared for each collection date.

Fish Oil Preparation

All fish oil was prepared by Lauren Ridley working in the laboratory of Dr. John Sowa of Seton Hall University. The head and tail of the menhaden fish were cut off, and the fish were deboned and fillet. The filets were cut into smaller pieces and pounded into a fish meal using a long glass tube inside a 40 mL round bottom centrifuge tube. The fish meal was centrifuged in the 40 mL round bottom centrifuge tube for six hours at 10,000 rpm. Following centrifugation, two top layers could be seen- one oil layer and one aqueous layer (Figure 2). The bottom of the 40 mL round bottom centrifuge tube was punctured to separate the two layers, and the fish oil was drained into a clean glass vial. Fish oils were preserved with nitrogen gas and stored in a -20 □C freezer. Each oil sample was made from five or more fish.

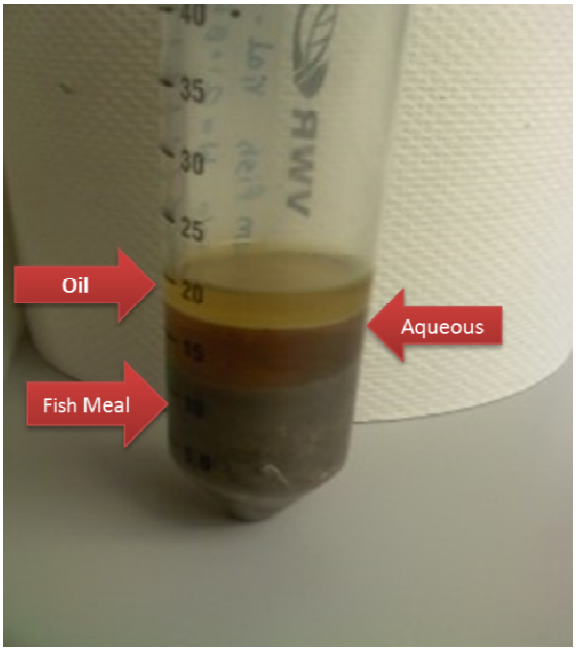


Figure 2. Layers generated by centrifugation of dissected fish material. Top layer shows the fish oil, middle layer consists of the aqueous solution and the bottom layer is the fish meal.

Preparation and Fluorescence Analysis of Fish Oils and Standards

To extract fluorescent compounds from fish oil, the fish oil was first thawed and mixed by vortexing. In a 1.5 mL microcentrifuge tube, 50 µl of fish oil and 1.15 ml of ethanol (EtOH) were combined. The mixture was vortexed continuously for 1 minute, and then the fish oil was separated from the mixture by centrifuging for 20 minutes at 13,000 rpm. The fish oil went to the bottom of the tube, and 1ml of the supernatant was removed and analyzed in a quartz cuvette. Different percentages of EtOH were used when developing the extraction method: they ranged from 50 to 100%.

Fish oil samples were analyzed for fluorescent compounds using two settings on a Spectramax M5 scanning fluorometer (Molecular Devices, CA). The first setting involved holding the emission (Em) wavelength at 350 nm and scanning for excitation (Ex) wavelengths from 250 to 340 nm. This setting was best for the detection of low molecular weight (LMW)

fluorescent compounds, such as vitamin E, and PAHs with two or three aromatic rings such as naphthol (2 rings) and fluorene (3 rings). The second setting involved holding the emission wavelength at 450 nm and scanning for excitation wavelengths from 250 to 440 nm. This setting was best for vitamin A and high molecular weight (HMW) PAHs such as hydroxypyrene (4 rings) and benzo[a]pyrene (5 rings). Relative Fluorescence Unit (RFU) values represented the intensity of excitation as detected by a particular emission wavelength (350 nm or 450 nm). The higher the RFU value, the greater the abundance of PAHs, vitamins or proteins detected.

Fish oil samples were also analyzed using a Fluorolog 3 (Horiba Jobin Yvon, Inc., Edison, NJ), which provided 3D contour maps showing excitation scans for multiple emission wavelengths. Fish oil samples were analyzed in a 1 ml quartz cuvette with the emission scanning from 320 to 480 nm and excitation scanning from 260 to 400 nm. The contour map values were represented in photon labeled counts per second per microamps (CPS/microamps). The contour maps displayed 3D data as heat maps with the red color representing high intensity and blue color representing low intensity fluorescence. The contour map separated fluorescence compounds based on their optimal emission and excitation wavelengths.

PAH standards were obtained from Sigma-Aldrich: benzo (a) pyrene-7-d, lot number 09720TG (BaP), fluoranthene, lot number MKBF9349V (FAN), phenanthrene, lot number BCBF4071V (PAN), 1-hydroxypyrene, lot number MKBD7640V (HPY), 9-phenanthrol, lot number BGBB3193V (HPA), 2-naphthol, lot number STBB7141V (HNP) and 2-hydroxyfluorene, lot number MKBG3676V (HFL). Other standards were also obtained from Sigma-Aldrich: bovine serum albumin, lot number 049K1585, α-tocopherol (vitamin E), lot number 100M1267V and retinol (vitamin A), lot number BCBK0033V. PAH standards, vitamin standards and albumin stocks were dissolved into EtOH. EtOH was 190 proof from KOPEC,

CAS# 64-17-5. EtOH was diluted with 18 Ohm water from Milli-Q Integral 5 (EMD Millipore Inc.).

Different concentrations of EtOH were used when analyzing percent recovery. PAH concentrations were 1250 ng/ml when generating spectra using the Spectramax M5 and 50 ng/ml using the Fluorolog 3. Concentrations for other standards were: 5,000 ng/ml for vitamin E, 25,000 ng/ml for vitamin A and 30,000 ng/ml for albumin. Detection limits for each compound were determined using standard curves. All PAH detection limits were 5 ng/ml. Detection limits for other standards were 10,000 ng/ml vitamin A, 1,000 ng/ml vitamin E, and 3,000 ng/ml albumin. Detection limits were the same for the Spectramax M5 and Fluorolog 3.

For fish oil percent recovery, there were three types of samples: fish oil only, fish oil with spike, and spike only. The fish oil alone sample was generated with 50 µl of fish oil extracted into a final volume of 1000 µl 75% EtOH. The fish oil spiked with PAH was generated by adding 4 µl of 25 µg/ml HNP or HPY stock in 75% EtOH to 50 µl fish oil and vortexing. To that, 946 µl of different percentages of EtOH was added for a final volume of 1 ml containing 100 ng/ml PAH if the entire spike was extracted into the EtOH. The HNP and HPY spike only sample was prepared by adding 4 µl of a 25 µg/ml stock to 996 µl of different percentages of EtOH for a final concentration of 100 ng/ml.

Concentrations of PAH-like compounds in fish oil were calculated from 2D fluorescence as follows. RFU values from the standard's major excitation peak at fixed emissions of 350 or 450 nm were converted into ng/ml using standard curves. Standard curves were generated for each standard using its major excitation peak at Em350 or Em450. The concentration in fish oil was then adjusted for percent recovery and divided by the average weight of the fish oils, 40 ± 1.5 mg. Final units were µg PAH-like compound/g fish oil. The fixed wavelengths used were

Ex270/Em350, Ex290/Em350, Ex280/Em350, Ex330/Em450 and Ex340/Em450 for HNP, vitamin E, albumin, vitamin A and HPY, respectively. The % recovery using 75% EtOH was 23, 4, 77.4, 30 and 66.3% for HNP, vitamin E, albumin, vitamin A and HPY, respectively. Linear line equations from standard curves were: HNP, y=4.2317x; vitamin E, y=0.0831x; albumin, y=0.0159x; vitamin A, y=0.1014x; and HPY, y=3.5235x.

Killifish Bile

Bile from menhaden could not be obtained; therefore, bile from killifish was provided by Frank J. Zadlock, IV working in the laboratory of Dr. Carolyn S. Bentivegna of Seton Hall University and Dr. Keith R. Cooper of Rutgers, The State University of NJ. Bile was obtained from killifish collected from an estuary; Little Sheepshead Creek at Tuckerton, NJ on July 19th, 2013. The killifish were acclimated to laboratory conditions for one week at Rutgers University, NJ. Macondo 252 (MC252) crude oil, collected from the DeepWater Horizon oil rig prior to the accident, was used to expose the fish twice on day 0 of the experiment using the gavage method. The killifish were sacrificed by euthanizing them on the third day (72 hours after gavaging) with an overdose of Tricaine Methanesulfonate (MS-222). The gall bladder was dissected out for SFS analysis using the bile content.

Results

The research focus was to determine whether high levels of PAHs can be detected in fish oil. Menhaden were chosen due to their common use in commercial products such as fish oil supplements, as well as their important role in the ecosystem as a major prey species that may transfer environmental contaminants to higher trophic levels. A Spectramax M5 was used to obtain 2D data and a Horiba Fluorolog 3 was used to obtain 3D data.

In fall 2010, menhaden were collected from NJ, VA and LA (Figure 1). Three collections were made of NJ fish. They were collected from different commercial ships seining along the NJ shore or off shore. One collection was made of LA and VA fish. The LA fish were caught off of Grande Terre Beach in Grand Isle. The VA fish were caught in the lower part of the James River near the mouth of the Chesapeake Bay. In the fall 2011, menhaden were collected at Barataria Bay, LA and Delaware Bay, NJ but not James River, VA. Another sampling site was added in LA, which was at Vermillion Bay, where less DWH oil came ashore compared to Barataria Bay. It was anticipated that VBLA might be a negative control for BBLA. Acronyms for the collections were based on collection location or ship: Barataria Bay, LA- BBLA, James River, VA- JRVA, Enterprise, NJ- EPNJ, Mt. Vernon, NJ- MVNJ, and Sea Huntress, NJ-SHNJ. Two collections of menhaden were obtained from the same two ships, Enterprise and Mt Vernon, and are referred to as EPNJ1/MVNJ1 and EPNJ2/MVNJ2.

Characteristics of Wild Menhaden

Morphological parameters were measured for the wild menhaden including their length (cm) and weight (g). A photograph of each fish was taken prior to dissection, so that the species could be identified. Results showed that the NJ fish were larger than the LA fish (Table 1). For example, the menhaden collected in LA range from 17.9-19.0 cm, while the menhaden collected in NJ range from 24.0-26.4 cm. Menhaden from LA weighed between 92-143 g, and menhaden from NJ weighed 279-355 g. This was primarily because the species collected in NJ were Atlantic menhaden, *B. tyrannus*, and those collected in LA were gulf menhaden, *B. patronus*, although there are no differences in species as far as accumulation of PAHs. Atlantic menhaden are known to be the larger of the two species (Nelson and Ahrenholz, 1986). Menhaden from VA were a mix of these two species.

Table 1. Morphological characteristics of menhaden used for fish oil. Table shows the state, site and more specific location of where the menhaden were collected. The date(s) of collection, number of fish and species are given. Species include *B. patronus* (P) and *B. tyrannus* (T). Average length (cm) and weight (g) of fish from a particular collection are shown with their standard deviation (SD). NJ fish are categorized by the seining ship from which they were obtained.

State	Site	Date	Amount	Species	Length Ave(SD)	Weight Ave(SD)
LA	Barataria Bay	10/30/2010	38	P	17.9 (3.3)	143 (57)
LA	Barataria Bay	7/28/2011	21	P	17.1 (1.8)	92 (27)
LA	Vermilion Bay	7/6/2011	20	P	15.1 (2.1)	64 (31)
VA	James River	10/[illegible]-21/2010	8	P	19.0 (2.7)	120 (43)
VA	James River	10/[illegible]-21/2010	10	T	22.0 (4.1)	195 (89)

State	Ship	Date	Amount	Species	Length Ave(SD)	Weight Ave(SD)
NJ	Enterprise1	9/8/2010	12	T	26.2 (1.7)	351 (50)
NJ	Enterprise2	9/21/2010	10	T	24.7 (1.8)	295 (50)
NJ	Mt Vernon	9/7/2010	11	T	26.4 (9.9)	324 (41)
NJ	Mt Vernon	9/23/2010	7	T	26.5 (10).3	355 (53)
NJ	Sea Huntress	10/18/2010	18	T	24.0 (2.2)	279 (75)

Information on the amount of fish oil obtained from menhaden tissues was determined by Lauren Ridley. Analyses showed that the most oil was collected from NJ fish, 3.56 grams of oil per fish, and the least from VA fish, 0.35 grams of oil per fish. The amounts of oil from LA were 1.22 grams of oil per fish (Table 2).

Table 2. Fish oil extraction data. Examples of information from aliquots of fish oil prepared from NJ, LA and VA menhaden collected in fall 2010. Oil was prepared from 5 fish collected from a particular location/ship. Weight (g) is the combined weight of the 5 fish and the total amount of oil (g) from those 5 fish. Also provided is the % of oil from the 5 fish and amount (g) and % of oil per fish. Please note that for Enterprise NJ 9 fish were used as opposed to 5. Not all fish oil extractions are shown.

#of Fish	Ship/Location	Weight of Fish (g)	Amount of Oil (g)	Total % Oil	Oil per Fish	% Oil per Fish
5	Mount Vernon NJ	1723	17.34	1.01	3.47	0.01
5	Grand Isle LA	826	6.35	0.77	1.22	0.007
5	James River VA	1191	1.729	0.15	0.35	0.001
9	Enterprise NJ	3205	32	1.01	3.56	0.01

Standards and Method Development

PAHs were analyzed at 1250 ng/ml in 75% EtOH using 2D SFS. The dependent variable (Y-axis) of a spectrum depicted fluorescent intensity units (RFU). Standards for 2-3 ring PAHs included: hydroxyfluorene (HFL), naphthol (HNP), phenanthrene (PAN) and phenanthrol (HPA), and standards with 4-5 ring PAHs included: benzo (a) pyrene (BaP), fluoranthene (FAN) and hydroxypyrene (HPY). In order to compare multiple PAHs, two types of fluorescent spectrum were established when using the Spectramax M5/M5 (Figure 3a, 3b). The first spectrum detected 2-3 aromatic rings holding the emission (Em) at 350 nm and scanning for excitation (Ex) from 250-340 nm. The second spectrum detected 4-5 aromatic rings holding emission at 450 nm and scanning from 250-440 nm.

PAHs usually showed two Ex peaks when emission was fixed at 350 nm (Figure 3a). For example, HFL showed one major excitation peak at 270 nm (5,340 RFU) and one minor peak at 310 nm (1,100 RFU). HNP showed less fluorescence intensity and a slightly different excitation pattern with its major peak also at 270 nm (3,800 RFU) and a second minor peak at 320 nm (1,964 RFU). HPA had a major peak at 250 nm (1,994 RFU) and a minor peak at 300 nm (415 RFU). The spectral patterns for these PAH standards were unique; however, some excitation wavelengths overlapped not allowing specific PAHs to be distinguished. When HNP or HFL were tested using a higher fixed emission of 450 nm, much lower fluorescence intensity was detected (Table 3). Therefore, compounds detected using Em350 were represented as HNP-like PAHs.

HMW PAHs exhibited more unique spectral patterns and greater fluorescence intensity in 2D scans by fixing the emission at 450 nm and scanning excitation from 250-340 nm (Figure 3a versus 3b and Table 3). Using fixed Em450, BaP had four peaks: 260 nm (7,493 RFU), 280 nm

(8,455 RFU), 360 nm (5,142 RFU) and 380 nm (4672 RFU). HPY had two peaks of similar intensity, 270 nm (4,329 RFU) and 340 nm (4,047 RFU). FAN had peaks at similar wavelengths; however, the one peak had greater fluorescence intensity than the other- Ex280 (6,477 RFU) and Ex350 (2,438 RFU). As with 2-3 aromatic ring PAHs, the spectral patterns were unique to the PAHs even though some shared one or more excitation peaks. The 4-5 ring PAHs had much higher fluorescence intensity at Em450 than Em350; therefore, compounds detected using Em450 were represented as HPY-like PAHs.

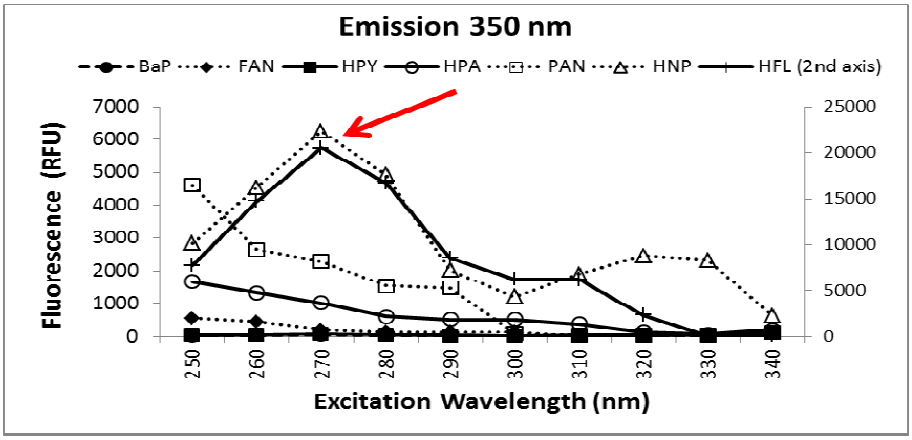


Figure 3a. Spectra of PAH standards at a 350nm fixed emission. Low MW PAHs fluoresced more strongly at EM350 than high MW ones. All PAH concentrations were 1250 ng/ml in 75% EtOH. PAHs analyzed were benzo(a)pyrene (BaP), fluoranthene (FAN), phenanthrene (PAN), hydroxypyrene (HPY), phenanthrol (HPA), naphthol (HNP) and hydroxyfluorene (HFL). The red arrows represent one major peak for HNP.

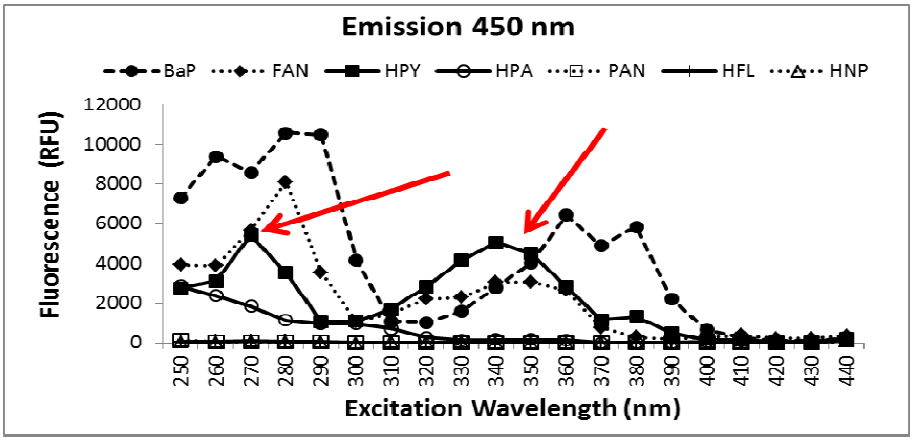


Figure 3b. Spectra of PAH standards at a 450nm fixed emission. High MW PAHs fluoresced more strongly at EM450 than low MW ones. All PAH concentrations were 1250 ng/ml in 75% EtOH. PAHs analyzed were benzo(a)pyrene (BaP), fluoranthene (FAN), phenanthrene (PAN), hydroxypyrene (HPY), phenanthrol (HPA), naphthol (HNP) and hydroxyfluorene (HFL). The red arrows represent two major peaks for HPY.

Table 3 provides the Ex and Em wavelengths of vitamins A&E and PAH standards using 2D Spectramax scans. The RFU values shown are at the Ex/Em providing maximum intensity (max) for a particular PAH and at the Ex/Em using fixed wavelengths of Em350 (naphthol-like PAHs) or Em450 (hydroxypyrene-like PAHs). Data showed that vitamins A&E and PAH standards have specific wavelengths for which they have maximum fluorescence. For example, BaP had a maximum at Em410/Ex280 (14,858 RFU). It could also be detected at Em450/Ex280 (5307 RFU). However, BaP was not detected at Em350/Ex280 (8 RFU). Vitamin E was detected at both Em330/Ex290 (765 RFU) and Em350/Ex290 (449 RU). However, Vit E was not detected at Em450/Ex290 (4 RFU). Moreover, HFL was detected at Em330/Ex270 (17,718 RFU) and Em350/Ex270 (11,255), but it was not detected at Em450/Ex270 (49 RFU). Overall, results showed that each PAH had a unique optimal Ex/Em; however, they could also be detected at the fixed emissions developed to detect them in fish oil. The fixed emission of 350 nm allowed vitamin E, HNP, HFL to be detected at 59-100 % of their optimal wavelengths. The fixed emission of 450 nm allowed vitamin A, HPY, FAN and BaP to be detected at 9-96% of their optimal wavelengths. While HPY detection was low (9%) at Em450, its RFU values were similar to those of the other HMW PAHs; so in a mixture, it could be detected using Em450.

Table 3. Excitation (Ex) and emission (Em) wavelengths of vitamins A&E and PAH standards. Fluorescence intensity (RFU) is shown at the Ex WL , Em WL providing maximum (max) intensity and at Ex-max using fixed wavelengths of Em350 (naphthol-like PAHs) and Em450 (hydroxypyrene-like PAHs). Ex = Excitation, Em = Emission, WL = Wavelengths, Vit A = vitamin A, Vit E = vitamin E, HNP= naphthol, HFL = hydroxyfluorene, HPY= hydroxypyrene, FAN = fluoranthene, BaP = benzo(a)pyrene.

			Fluorescence Intensity (RFU)		
Compound	Ex WL-max	Em WL-max	Ex WL/Em WL-max	Ex-max/Em350	Ex-max/Em450
Vit A	325	470	343	0	280
Vit E	290	330	765	449	4
HNP	270	350	3704	3704	67
HFL	270	330	17,718	11,255	49
HPY	270	390	28,000	34	2664
HPY	340	390	28,248	NA	2665
FAN	280	460	3032	32	2917
BaP	280	410	14,858	8	5307

The effects of vitamins on PAH spectra were analyzed by testing them alone and in combination with HNP or HPY (Figure 4a and 4b). Results showed a major peak for vitamin E at Em350/Ex290 when tested alone. Interestingly, vitamin E spiked with HNP demonstrated a major peak at 280 nm excitation when holding the emission at 350 nm (Figure 4a). This major peak was where most of the wild-caught fish oils fluoresced in 2D scans as shown in later figures. Therefore, what was seen in fish oil may have been a combination of HNP and vitamin E. Results for vitamin A showed a major peak at Em450/Ex330 (Figure 4b). However, when combined with HPY, the major peaks of HPY increased at Ex270 and Ex340 even though neither of those peaks was found for vitamin A. In addition, there was a small increase between Ex310 and 330 nm, which was where vitamin A fluoresced on its own. 2D scans of some fish oils did show a vitamin A-like peak at Em450/Ex330 as shown in later figures.

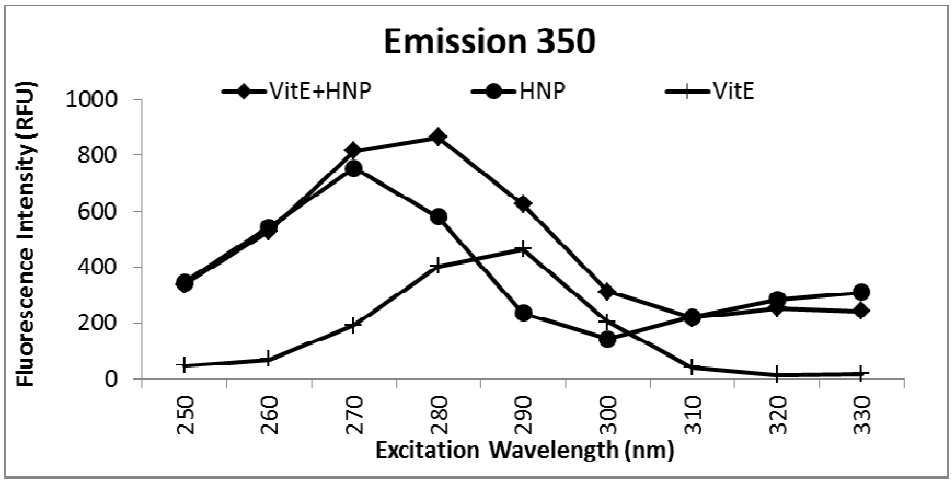


Fig 4a. Fluorescent spectra for Vitamin E (Vit E) with or without Naphthol (HNP) at 350 nm emission. Separate peaks for Vitamin E and HNP merged into one peak at Em350/Ex280: this peak was similar to the one seen in fish oil and for albumin. All samples were extracted into 75% EtOH, Vitamin E =5,000ng/mL, and HNP=100ng/mL.

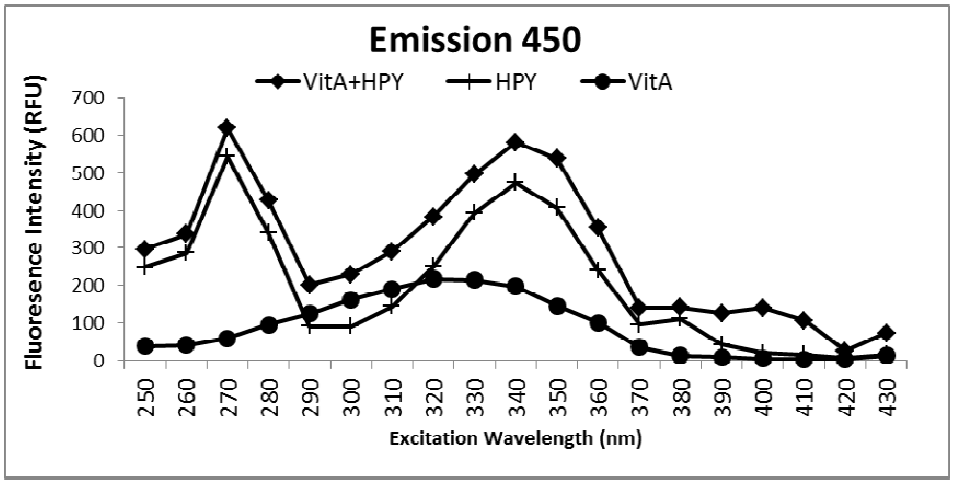


Figure 4b. Fluorescent spectra for Vitamin A (Vit A) with or without Hydroxypyrene (HPY) at 450 nm emission. Spectra show that combining Vit A and HPY only modestly alters their individual spectrums with a slight increase at Ex310-320. All samples were extracted into 75% EtOH, Vitamin A = 25,000 ng/ml, HPY = 100 ng/ml.

The Fluorolog 3 demonstrated how a 3D scan can better distinguish two or more fluorescent compounds on a contour map than a 2D scan. A contour map represents fluorescence peaks using a heat design. The red demonstrates high fluorescence intensity, while the blue demonstrates low fluorescence intensity. Figure 5 shows a contour map with one or two standards on the same map. As expected, HNP showed a major peak with a maximum at

Em346/Ex270 ($1e^{+5}$ CPS). HPY showed four fluorescence peaks: Em395/Ex260-275 ($2e^{+6}$ CPS), Em395/Ex320-360 ($1e^{+5}$ CPS), Em410/Ex265-275 ($2e^{+5}$ CPS), and Em410/Ex320-360 ($5e^{+5}$ CPS). Vitamin A showed one fluorescence peak at Em425-480/Ex310-345 ($1e^{+5}$ CPS), and vitamin E showed one fluorescence peak at Em350/Ex290 ($8e^{+5}$ CPS). Albumin also showed fluorescence where expected with a peak at Em350/Ex280 ($1e^{+5}$ CPS). The Fluorolog 3 data coincided with the Spectramax M5 data showing similar emission and excitation fluorescent peaks. Results for the Fluorolog 3 also showed the difficulty in distinguishing vitamin E, HNP and albumin from one another because each compound had fluorescence at similar emission and excitation wavelengths (Figure 5). For example, HNP was excited at 270 nm, Vit E at 290 nm and albumin at 280 nm, and their emission ranged from 320-370 nm. This similar range of Ex/Em produced overlapping contour waves making it difficult to identify individual compounds when mixed together. HPY and vitamin A were more easily distinguished as their optimal emission wavelengths were farther apart. The contour maps provided much better distinction between fluorescent compounds because it provided both optimal emission and excitation wavelengths.

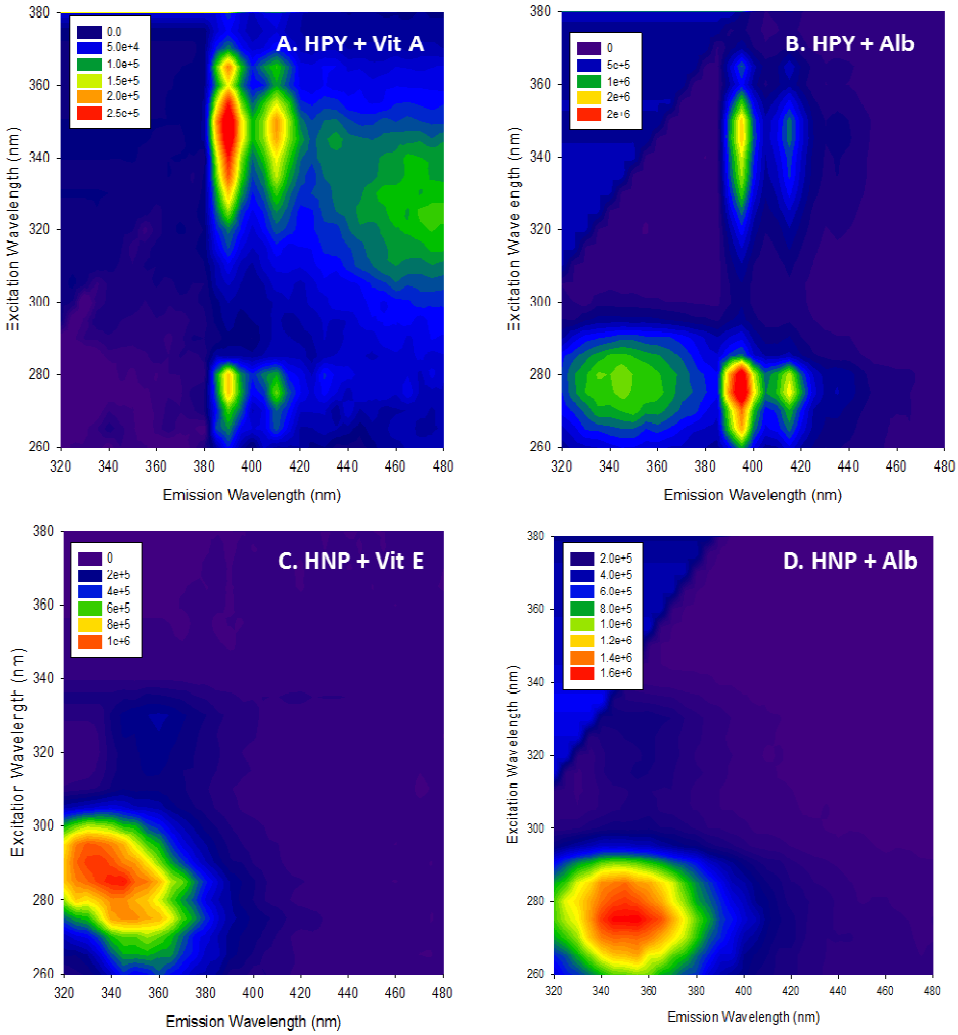


Figure 5. Contour maps of 3D fluorescence spectra for standards extracted into 75% EtOH. The 3D spectrum results from scanning excitation at multiple emission wavelengths. Red color represents the highest fluorescent intensity. A. Shows a solution with HPY and VitA. HPY fluoresces at two different spots- Ex270 and Ex340/Em400-420, while vitamin A fluoresces at Ex320-340/Em440-480. B. Shows a solution with albumin (Alb) and HPY. Alb fluoresces at Ex280/Em340-360. C. Shows a solution with vitamin E (VitE) and HNP. HNP fluoresces at Ex270/Em350 and VitE at ex290/Em340-350. D. Shows a solution with Alb and HNP, which cannot be distinguished from one another. Note that this 3D data correlates with SpectraMax M5 data seen in 2D graph. Alb = Albumin at 30,000 ng/ml, HPY = Hydroxypyrene at 50 ng/ml, HNP = Naphthol at 50 ng/ml, VitA = Vitamin A at 25,000 ng/ml, and VitE = Vitamin E at 5,000 ng/ml

EtOH concentration was an important factor in the method development of this assay because EtOH can extract different amounts of a compound based on its hydrophobicity as well as affect the fluorescence intensity of a compound. Results for 2D scans showed that EtOH concentration can intensify the fluorescence of both vitamins A and E. For example, vitamin E

analyzed in 50% EtOH provided a fluorescence intensity of 50 RFU (Em350/Ex290), while 75% EtOH provided fluorescence intensity of 302 RFU (Em350/Ex290) (Figure 6a). Correspondingly, vitamin A analyzed in 50% EtOH provided a fluorescence intensity of 93 RFU (330Ex/450Em), while 75% EtOH provided fluorescence intensity of 320 RFU (330Ex/450Em) (Figure 6b).

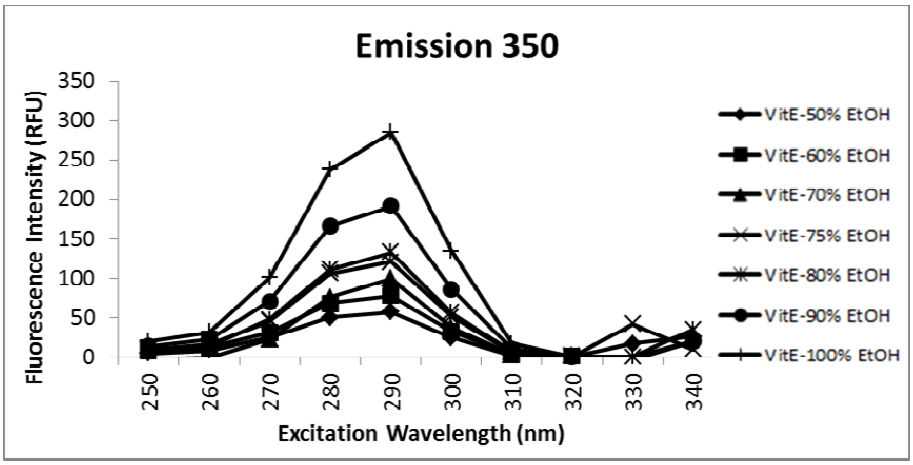


Figure 6a. Effect of EtOH concentration (%EtOH) on Vitamin E (Vit E) extraction. Spectra show increasing levels of extraction of vitamin E with increasing percentages of ETOH solvent. The Vit E concentration was 1250 ng/ml. Vit E has poor fluorescence at EM450: data not shown.

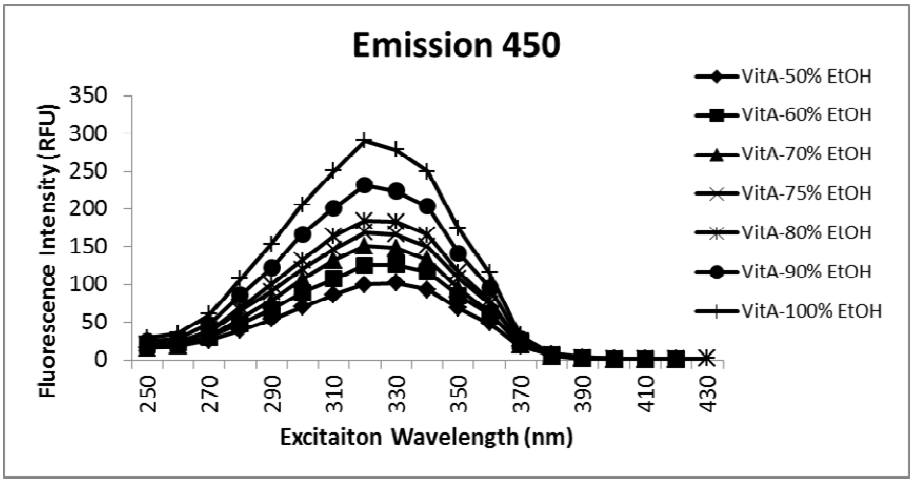


Figure 6b. Effect of EtOH concentration (%EtOH) on Vitamin A (Vit A) extraction. Spectra show increasing levels of extraction of vitamin A with increasing percentages of ETOH solvent. The Vit A concentration was 1250 ng/ml. Vit A has poor fluorescence at EM350: data not shown.

Since EtOH concentration appeared to affect the detection of fluorescent compounds, the percent recovery of vitamins, PAHs and albumin from fish oil was determined by extracting them into different concentrations of EtOH. Standards were spiked into menhaden fish oil from DayBrook Industry (DB) and extracted into different ethanol concentrations ranging from 50-100% (Table 4). Recovery of naphthol (HNP- 100 ng/ml) was higher when extracted by 50% than 100% EtOH; conversely, recovery of hydroxypyrene (HPY- 100 ng/ml) was higher when extracted by 100 % at Em450/Ex340 (89.1% recovery) than 50% at Em450/Ex340 (22.8% recovery) EtOH. Comparing percent recoveries for the various standards supported the use of 75% EtOH as a concentration that could extract both more hydrophilic and hydrophobic compounds with at least 30% recovery for all compounds except vitamin E. Very little Vit E was recovered from the spikes. Recovery of Vit E when extracted in 75% EtOH was 4% (Em350/Ex290), while in 100% EtOH it was 12% (Em350/Ex290). Loss of vitamins/PAHs may have occurred due to the spiked actually dissolving into the fish oil and then being removed from the supernatant upon centrifugation. The spike went with the fish oil to the bottom of the tube such that less was recovered in the supernatant.

Table 4. Percent recovery of PAHs and vitamins spiked into DayBrook fish oil and extracted into different concentrations of EtOH. Vit A = vitamin A, Vit E = vitamin E, HNP= naphthol, HPY= hydroxypyrene, DB= DayBrook Fish Oil. Vit E = 5000 ng/ml, Vit A= 25,000 ng/ml, PAHs = 100 ng/ml, Alb = 30,000 ng/ml.

		EtOH Concentrations						
CMPD	Wavelengths	50%	60%	70%	75%	80%	90%	100%
VitE+DB	Ex290/Em350	17	7	4	4	4	9	12
VitA+DB	Ex330/Em450	17	19	34	30	26	38	30
HNP+DB	Ex270/Em350	35	33	30	23	19	9	2
Alb+DB	Ex270/Em350	70.9	75.5	56.4	77.4	35.7	43.7	21.7
HPY+DB	Ex270/Em450	10.5	14.4	19.3	15.3	13.9	7.8	-1.2
HPY+DB	Ex340/Em450	22.8	36.6	56.8	66.3	72.8	76.6	89.1
HPY	Ratio Ex270/Ex340	0.46	0.39	0.34	0.23	0.19	0.10	<0.001

The spectra for PAHs spiked in fish oil showed the effects of EtOH on extraction. For HNP spiked into DB fish oil, extraction of HNP appeared greater with 50% than 75% EtOH. This was shown by a shift in the major peak at Em350/Ex280 using 75% EtOH to a major peak at Em350/Ex270 using 50% EtOH (Figure 7a). The major peak at Em350/Ex270 was characteristic of HNP only. Changes in spectra were also seen when extracting HPY from DB fish oil. The Em450/Ex340 peak was characteristic of HPY only. This peak was 5x higher using 100% EtOH than 50% EtOH indicating better extraction of HPY using 100% EtOH (Figure 7b). The spectrum using 100% EtOH also indicated extraction of vitamin A from the DB fish oil. This was shown by an increase in the Em450/Ex320 peak seen in DB fish only when the sample was extracted using 100% EtOH. This peak was not characteristic of HPY and was at the excitation wavelength associated with vitamin A. This indicated the presence of vitamin A in DB fish oil. Another change in the HPY spiked spectrum was suppression of the Ex270 peak by the DB fish oil matrix. The HPY peak at Ex270 decreased at 100% EtOH while the one at Ex340

increased. This could have been due to interaction between the solvent and the levels of HPY and/or Vit A.

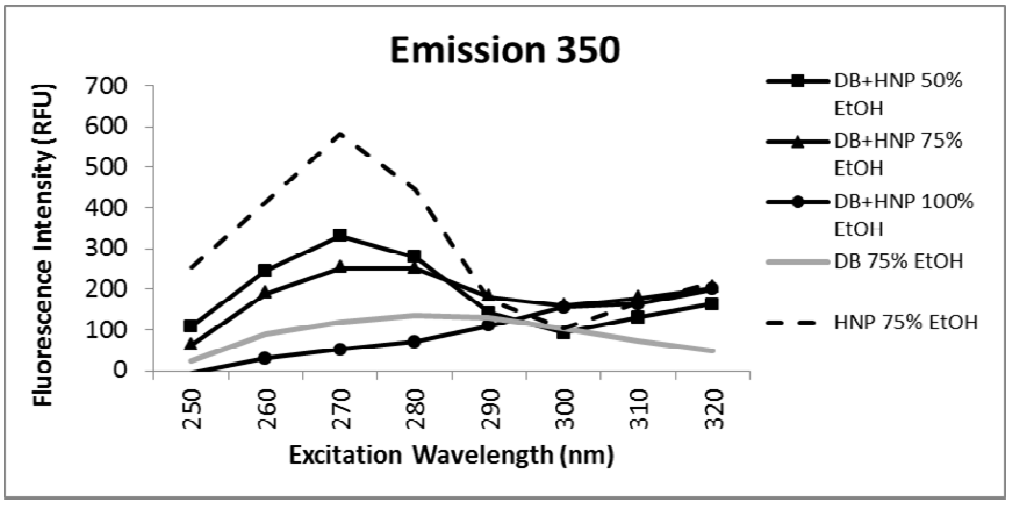


Figure 7a. Effect of EtOH concentration (%EtOH) on extraction of low MW PAHs from fish oil. More naphthol (HNP- 500 ng/ml) was extracted by 50% than 100% EtOH. The shift in peaks from Em350/Ex270 toward Em350/Ex280 indicated increased extraction of vitamin E at 75% compared to 50% EtOH. The entire oil sample dissolved into solution at 100% releasing unknown fluorescent compounds. DB= menhaden fish oil from Day Brook Industry.

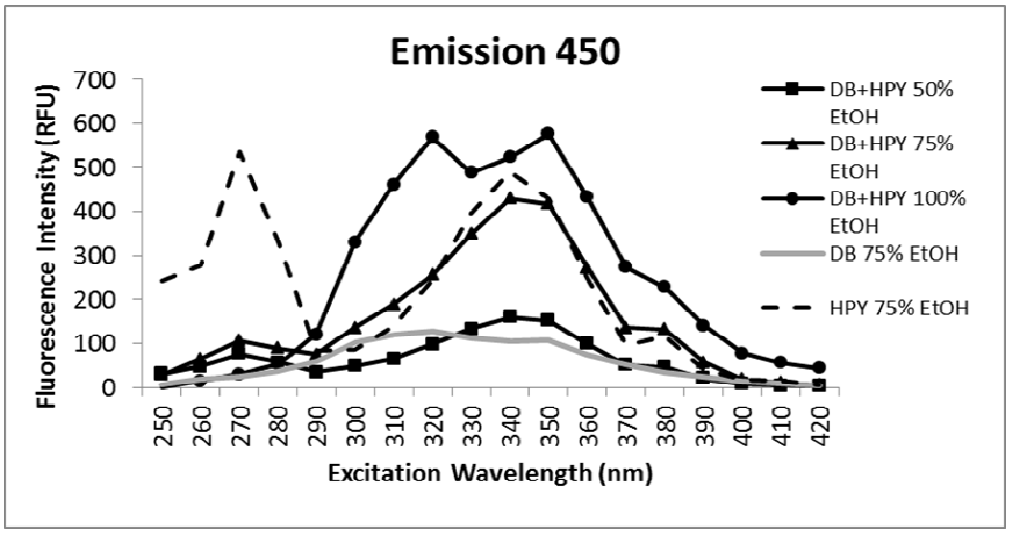


Figure 7b. Effect of EtOH concentration (%EtOH) on extraction of high MW PAHs from fish oil. More hydroxypyrene (HPY- 500 ng/ml) was extracted by 100 % than 50% EtOH. The vitamin A peak (Em450/Ex320) and HPY-like peak (Em450/Ex350) in un-spiked DB became more apparent at 100% ETOH indicating the presence of these compounds in DB. Note suppression of Ex270 by fish oil, this could be due to excimers formation by high HPY and/or vitamin A concentrations in extract. DB= menhaden fish oil from Day Brook Industry.

Different concentrations of EtOH appeared to extract different compounds from fish oil samples. This was seen when NB fish oil was extracted into 50, 75 or 100% EtOH. Results showed Vit E-like compounds when analyzed in 75% EtOH and HNP-like fluorescence when analyzed in 50% EtOH. This was shown by the major peak shifting from Em350/Ex290 using 75% EtOH to Em350/Ex270 using 50% EtOH (Figure 8a). Analyses using fixed emission of 450 nm also showed effects of EtOH concentration on NB fish oil. NB fish oil showed an Em450/Ex270 peak that was high when extracting with 75% EtOH and low when extracting with 100% EtOH. The change was similar to what was seen when comparing spectra for 50 and 100% EtOH for DB samples spiked with HPY (Figure 8b).

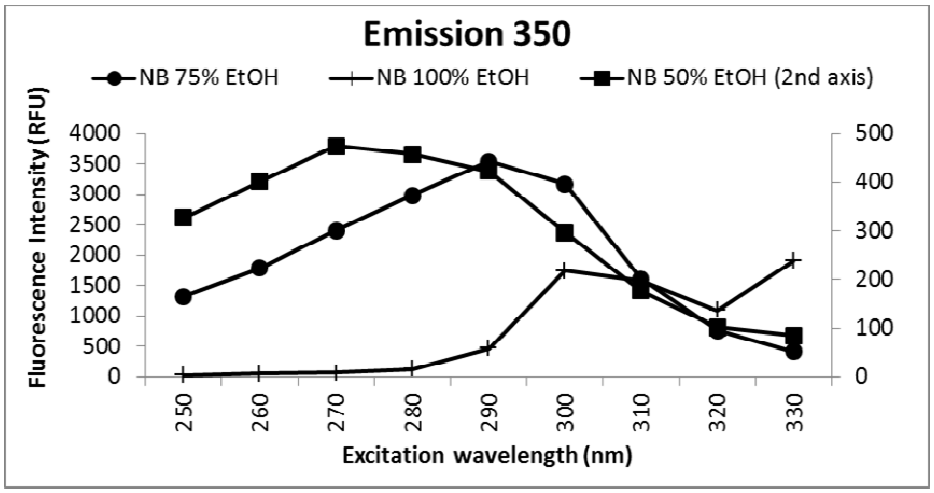


Figure 8a. Effect of EtOH concentration (%EtOH) on extraction of HNP-like PAHs from Nature's Bounty fish oil at 350 nm emission. The major peak of Em350/Ex270 appeared to result from a combination of HNP-like PAHs and vitamin E as increasing % EtOH shifted this major peak towards Em350/Ex290-300 indicating more vitamin E extraction at high % EtOH. 100% ETOH dissolved the oil sample and released unknown fluorescent compounds.

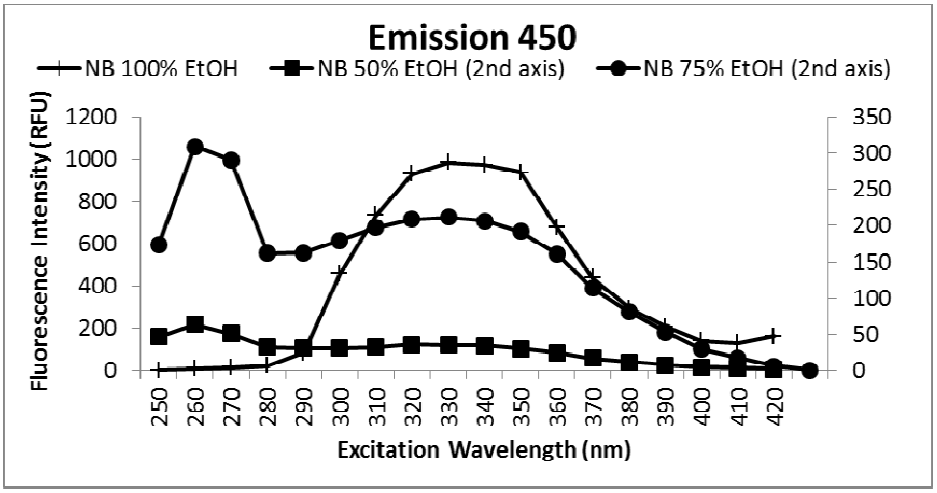


Figure 8b. Effect of ethanol concentration (EtOH %) on extraction of HPY-like PAHs from Nature's Bounty fish oil at 450 nm emission. Higher % EtOH appeared to extract more vitamin A (Ex320-330) as well as HPY-like PAHs (Ex340-350).

Menhaden Fish Oils and Killifish Bile Analyzed by Spectramax (2D spectra)

The Spectramax M5 generated 2D spectra for fish oils and killifish bile. Commercial fish oils showed a major peak at Em350/Ex290 and Em450/Ex330 demonstrating Vit E and Vit A, respectively (Figure 9a and 9b). In addition, NB fish oil showed some fluorescence at Em350/Ex270 suggesting the presence of HNP-like compounds. The Em 450 nm spectrum for

NB also varied from the other commercial fish oils by showing a major peak at Em450/Ex270. This indicated HPY-like compounds (Figure 9b). Among commercial fish oils, NB fish oil appeared to contain the most Vit E. For instance, NB fish oil had 3300 RFU at Em350/Ex290, while NM fish oil had 414 RFU at Em350/Ex290. GNC fish oil appeared to contain the most Vit A. Interestingly, GNC fish oil contained rosemary leaf extract, which might have accounted for the apparently high levels of vitamin A as detected using 2D analysis. Lovaza fish oil also had a very high Vit A peak, having 804 RFU at Em450/Ex330, when compared to NM and SV commercial fish oils. Lovaza had a second peak at Em450/Ex350 suggesting the presences of HMW PAHs in this prescription-level fish oil tablet. For the most part, all commercial fish oils seemed to only have peaks indicative of the presence of vitamins, with the exception of NB and Lovaza fish oils, which contained peaks, associated with PAH fluorescence.

Fish oil from wild menhaden collected in NJ in fall 2010 showed some peaks that were similar to those of commercial fish oils (Figures 10a and 10b). They had a major peak at Em350/Ex280. Compared to standards, this matched albumin-like fluorescent the best. Interestingly, MVNJ fish oil displays an Ex peak shifted towards 270 nm (413 RFU) (Figure 10 a), which suggested the presence of a naphthol-like compound as seen in NB fish oil. SHNJ fish oil appeared to have a major peak at Em350/Ex290 (48 RFU) suggesting vitamin E detection. All NJ fish oils (SHNJ, EPNJ-1, EPNJ-2, and MVNJ) showed one of their major peaks at 330 nm excitation when holding the emission at 450 nm. This suggested the presence of vitamin A. This peak occurred in all of the fish oils except JRVA. All NJ fish oil samples also had another peak at Em450/Ex350. This peak was higher than the 330 nm peak in MVNJ and was the single largest peak in JRVA. This peak was consistent with the presence of HMW PAHs in these samples when compared to PAH standards (Figure 3b). The 350 nm excitation peak in MVNJ

was approximately >2x higher than in SHNJ, EPNJ1, and EPNJ2 samples. This peak was approximately >3x in JRVA compared to all of the other samples. This indicated that MVNJ and JRVA samples had the most HPY-like PAH contamination in their fish oil.

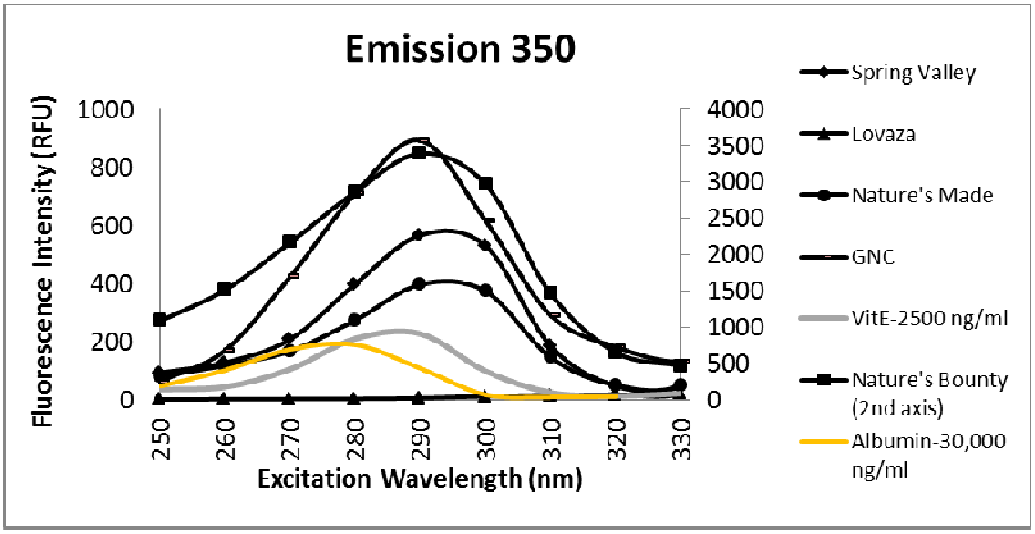


Figure 9a. Spectra of commercial fish oils and one prescription fish oil (Lovaza) at 350 nm emission. The spectra show vitamin E in fish oils when extracting with 75% EtOH at 290 nm excitation. Nature's Bounty commercial fish oil showed a very high vitamin E peak. Note vitamin E and albumin only fluoresce strongly at 350 nm emission.

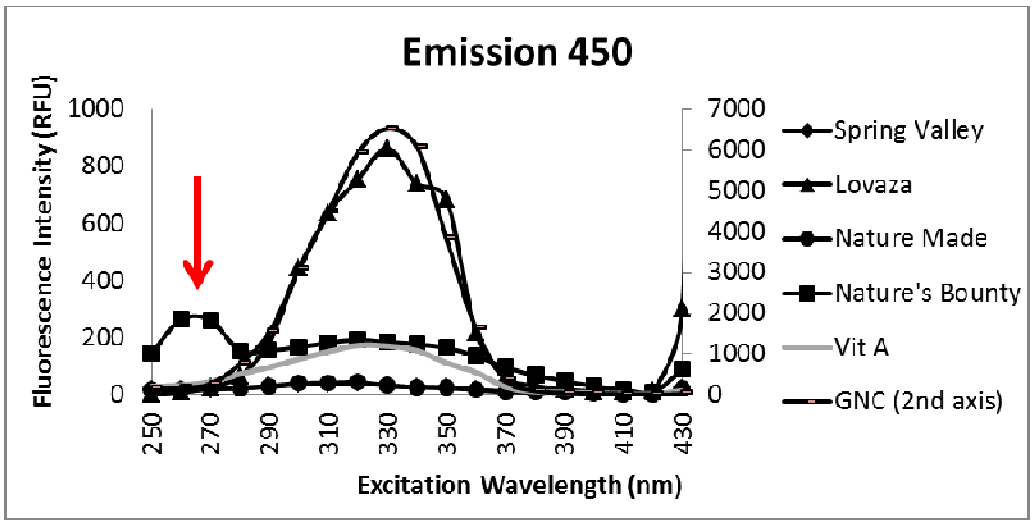


Figure 9b. Spectra of commercial fish oils and one prescription fish oil (Lovaza) at 450 nm emission. The spectra show vitamin A in fish oils when extracting with 75% EtOH at 330 nm excitation. Vitamin A appeared to be very high in GNC fish oil, which indicated that it is fortified with vitamin A. The red arrow represents another fluorescent compound in Nature's Bounty that may possibly represent PAHs. Note that vitamin A only fluoresces strongly at 450 nm emission.

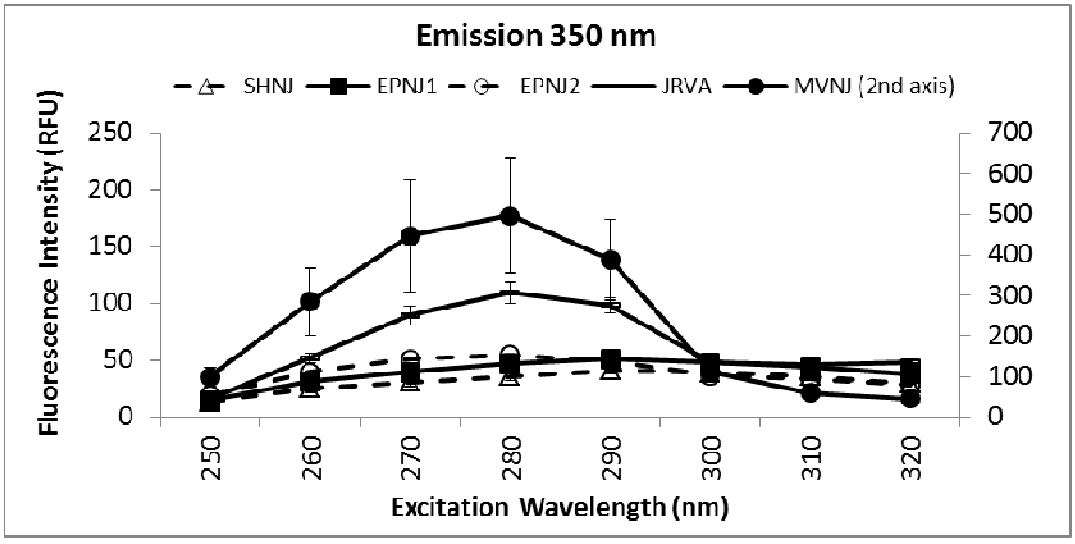


Figure 10a. Fluorescence spectra of wild fish oil at 350 nm emission. The spectra showed albumin in fish oils at a 280 nm excitation when extracting with 75% EtOH. The spectra also showed a vitamin E peak at 290 nm excitation. MVNJ fish oil had a very high albumin peak. In addition, MVNJ fish oil had PAHs –like fluorescence with its peak rising at 270 nm excitation. Error bars represent triplicate analyses of the same sample.

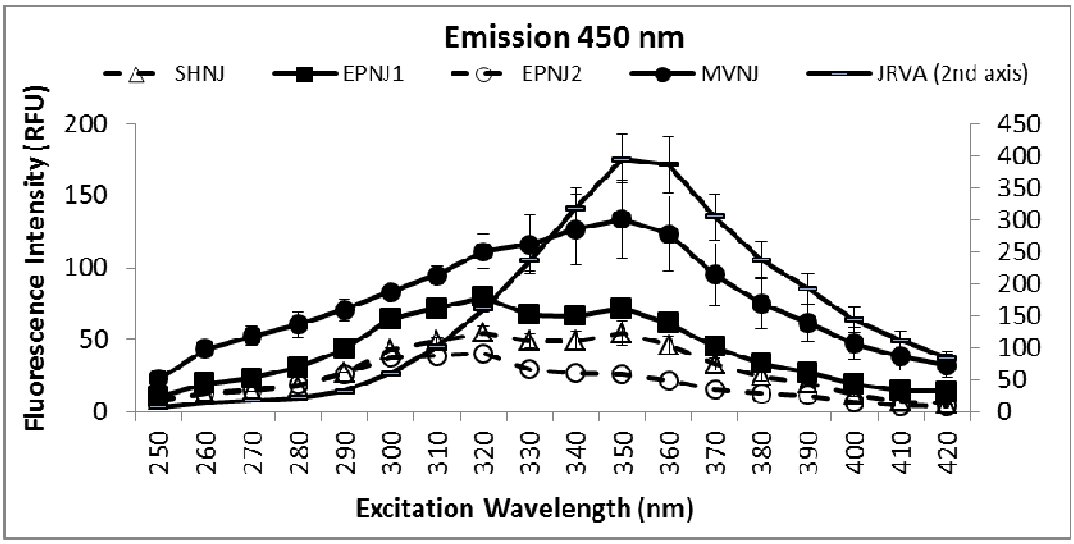


Figure 10b. Fluorescence spectra of wild fish oil at 450 nm emission. The spectra showed a 330 nm excitation peak for vitamin A and a 340-350 nm excitation peak for PAH-like compounds in fish oils when extracting with 75% EtOH. MVNJ fish oil appeared to have the greatest amount of high MW PAHs among the NJ samples. JRVA fish oil had levels 3x those of Atlantic coast samples. Error bars represent triplicate analyses of the same sample.

Results for 2D spectra showed similarities between wild menhaden fish oil and bile from killifish experimentally exposed to crude oil in the laboratory (Figure 11a and 11b). Fish oil collected from BBLA in fall of 2010 as well as VBLA in summer 2011 both had a single major peak at 280 nm excitation when holding emission at 350 nm. The killifish control bile had the same peak; although, the intensity was approximately 4x higher. Interestingly, bile collected from killifish gavaged with crude oil showed a fluorescence spectrum similar to that of BBLA spring 2011 fish oil with a major peak at Em350/Ex310 for both. BBLA fish oil collected in spring 2011 showed a HPY-like fluorescence similar to killifish gavaged with crude oil with a major peak at Em450/Ex350 (Figure 11b). Results for BBLA fall, 2010 and spring, 2011 indicated that PAHs in the spring were higher than in the fall as BBLA 2010 had a much lower RFU values than BBLA 2011. VBLA 2011 had higher levels of HNP-like PAHs and lower levels of HPY-like PAHs than BBLA 2011. 2D spectra of standards suggested that the presence of other fluorescent-like compounds such as vitamin E and protein might have increased levels of HNP-like compounds while HMW PAHs increased HPY-like PAHs. Overall, results indicated that crude oil like compounds were at higher levels in BBLA 2011 than VBLA 2011 fish oil.

Concentrations of fluorescent compounds in commercial and menhaden fish oils were calculated. The concentrations were calculated using fixed Em/Ex wavelengths and standard curves (Table 5). Fixed wavelengths were based on the major peak of the standard using either Em350 or Em450. Results indicated that NB fish oil had the highest HNP-like content at Em350/Ex270 (49 µg/g), the highest Vit E-like content at Em350/Ex290 (1904 µg/g) and the highest albumin-like content at Em350/Ex280 (4514 µg/g). NM fish oil had the second highest albumin-like content at Em350/Ex280 (1601 µg/g). GNC fish oil had the highest Vit A-like content at Em450/Ex330 (6365 µg/g) and the highest HPY-like content at Em450/Ex340 (80

µg/g). Concentrations of fluorescent compounds in menhaden fish oils indicated that MVNJ fish oil had the highest HNP-like content (2.52 µg/g), the highest Vit E-like content (102 µg/g) and the highest albumin-like content (598 µg/g). VBLA had the second highest albumin-like content (259 µg/g). JRVA had the highest HPY-like content (2.79 µg/g) and the highest Vit A-like content (57 µg/g). Comparison of spectra for GNC (Figure 9b) and JRVA (Figure 10b) showed how the broadness of the fluorescence peak can lead to the incorrect measurements of HPY-like PAHs in GNC and Vit A-like compounds in JRVA when using fixed wavelengths.

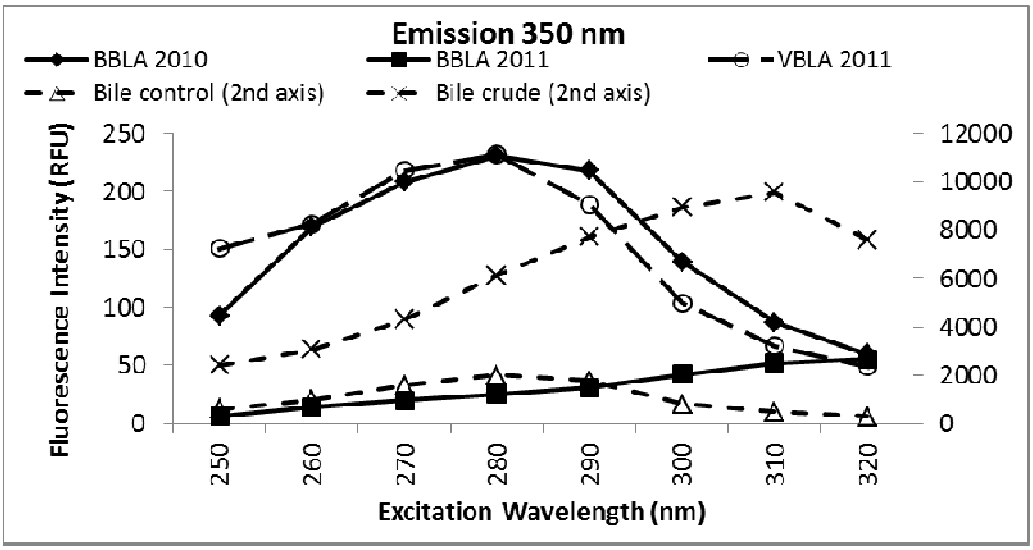


Figure 11a. Fluorescence spectra at 350 nm emission of wild fish oil from Barataria Bay LA (BBLA) and Bile from killifish exposed to crude oil (Bile crude). The spectra indicated the presence of albumin and/or vitamin E in BBLA 2010, VBLA 2011 and bile from unexposed fish (Bile Control) at 280 nm excitation. The spectra also show that fish oil from different years had different levels of HNP-like PAHs. The BBLA 2011 fish oil spectrum looked similar to that for Bile crude. Both showed a peak at 310 nm excitation indicating the presence of PAH contamination due to crude oil exposure.

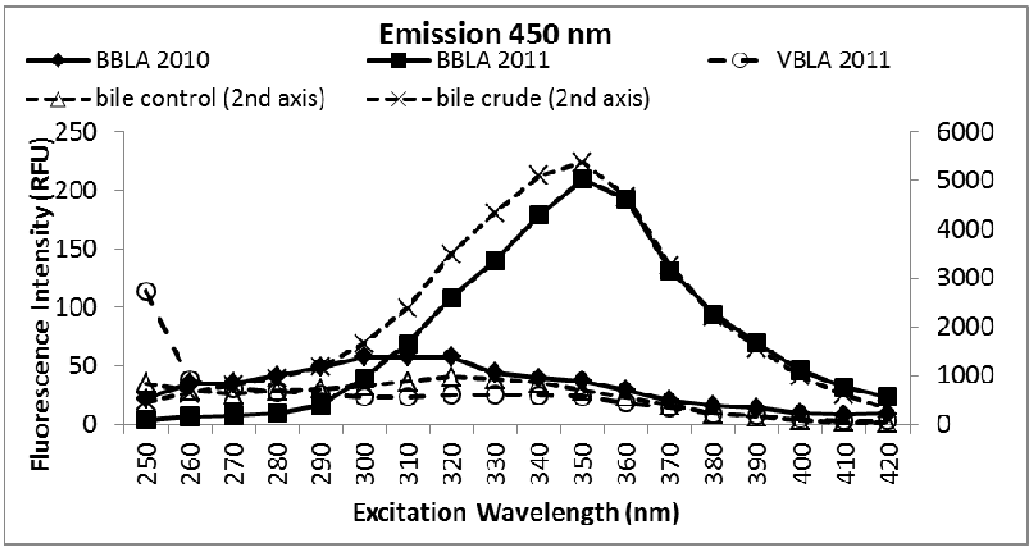


Figure 11b. Fluorescence spectra at 450 nm emission of wild fish oil from Barataria Bay LA (BBLA) and bile from killifish exposed to crude oil (Bile crude). The spectra show low levels of HPY-like PAHs for BBLA 2010 and bile control compared to BBLA 2011 and Bile crude. The spectra also showed that fish oil from different years had different levels of HPY-like PAHs. The BBLA 2011 fish oil spectrum looked similar to that for Bile crude. Both showed a peak at 350 nm excitation indicating the presence of PAH contamination due to crude oil exposure. VBLA 2011 had lower levels of HPY-like PAHs than those collected from BBLA at a similar time. HPY-like PAHs were 30x higher in Bile crude than BBLA 2011 fish oil.

Table 5. Extraction of different compounds from fish oil in 75% EtOH. The table shows the concentration of different compounds extracted from fish oil. Data is represented in µg/g of fish oil. Note that concentration detection limits were < 0.005 µg/g for PAHs, < 1µg/g for Vitamin A&E, and < 3 µg/g for Albumin.

		Fluorescent Intensity (µg/g of fish oil)			
Commercial F.O	HNP-Like	HPY-Like	Vit E-Like	Vit A-Like	Albumin-Like
Lovaza	1.68	3.55	291.37	357.8	482.83
Spring Valley	< 0.005	0.71	0.28	53.34	< 3
Nature's Made	8.92	0.38	865.71	27.3	1600.55
GNC	7.76	80.2	420.88	6365.18	907.65
DayBrook	1.97	0.9	72.04	71.52	197.24
Nature's Bounty	48.85	1.85	1903.62	140.54	4514.18
		Fluorescent Intensity (µg/g of fish oil)			
Wild Caught Menhaden F.O	HNP-Like	HPY-Like	Vit E-Like	Vit A-Like	Albumin-Like
JRVA	0.11	2.79	15.01	56.63	67.62
BBLA 2010	0.81	0.26	51.11	9.04	257.87
BBLA 2011	< 0.005	1.25	< 1	32.71	< 3
VBLA 2011	0.87	0.16	42.38	4.36	259.44
SHNJ	< 0.005	0.37	< 1	10.28	< 3
EPNJ1	< 0.005	0.49	0.87	14.96	< 3
EPNJ2	< 0.005	0.16	0.27	5.59	< 3
MVNJ	2.52	0.93	102.25	27.04	597.50

Menhaden Fish Oil, Crude Oil and Killifish Bile Analyzed by Fluorolog 3 (3D Spectra)

Three of the commercial fish oils were analyzed on the Fluorolog 3. The effect of EtOH was investigated by extracting them into 75 or 90% EtOH (Figure 12). For NB fish oil, the 75% EtOH appeared to extract vitamin E with maximum fluorescence at Em340/Ex295 as well as HNP-like PAHs with fluorescence extending to Em330/Ex270. Extraction with 90% EtOH appeared to extract only vitamin E. These results supported the 2D spectra generated by fixing emission at 350nm. The 2D spectra showed a maximum peak at Ex290 when extracting with 75% EtOH. There was also a shoulder at Ex270 indicative of HNP-like PAHs. Increasing the EtOH concentration to 90% shifted the peak of NB more toward Ex300 as seen in the 3D

spectrum (Figure 8a). Interestingly, the 3D spectrum for NB showed no vitamin A. This differed from the 2D spectrum using Em450, in which there appeared to be an Em450/Ex320 peak that was characteristic of the vitamin A standard (Figure 8b). For Lovaza, the 75% EtOH spectrum showed vitamin E at Em350/Ex295 and unknown fluorescent compounds at Em430/Ex310. The 90% EtOH extracted a vitamin A-like compound not seen when using 75% EtOH. These results differed from the 2D spectrum using Em350, in which there was no Vitamin E at Em350/Ex290 (Figure 9a). However, results supported the 2D spectrum using Em450 which showed a huge vitamin A peak at Em450/Ex330 that was also seen on the 3D spectra (Figure 9b versus Figure 12). NM appeared to contain only vitamin E (Em330/Ex295), which was extracted better by 90% than 75% EtOH as noted by the increased fluorescence intensity. These results supported the 2D spectra generated by fixing the Em350 and the Em450; such that, NM strongly fluoresced at Em350, but did not fluoresce at Em450 (Figure 9a and 9b). NM also appeared to only contain Vitamin E (Em350/Ex290).

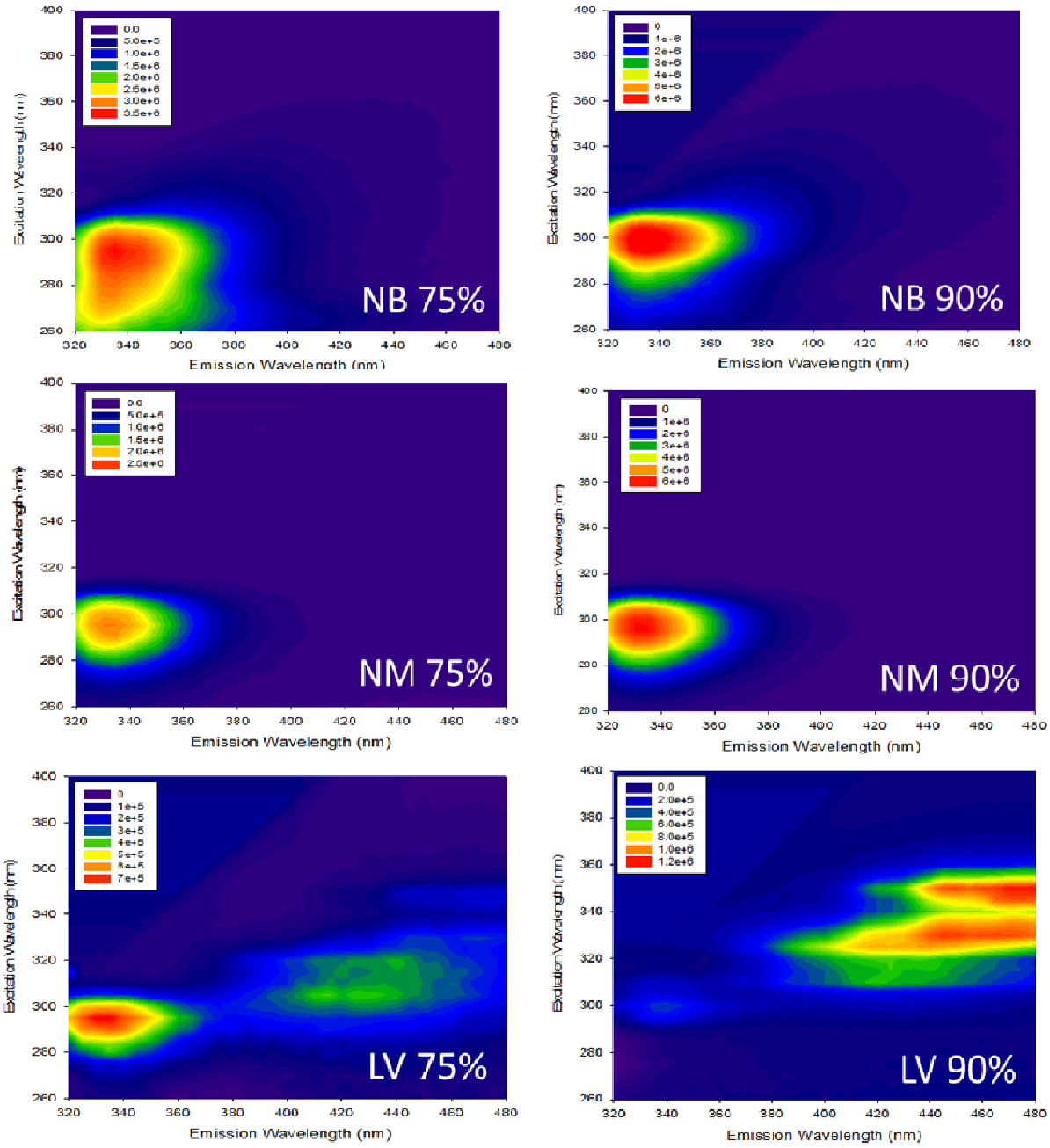


Figure 12. Contour maps of 3D fluorescence spectra for commercial fish oil extracted into 75% or 90% EtOH. NB= Nature's Bounty, NM= Nature Made, LV= Lovaza. Data show that different % EtOH can extract different fluorescent compounds as shown by the occurrence of new spots and/or shifts of existing ones. For NB, 50% EtOH appeared to extract HNP-like PAHs, while 90% EtOH appeared to extract vitamin E. Note the lack of vitamin A in NB. At 50% EtOH, Lovaza showed unknown fluorescent compounds (Ex310/Em430), while at 90% EtOH a vitamin A-like compound was extracted. NM appeared to contain only vitamin E (Ex290/Em330), which was extracted better by 90% than 75% EtOH as noted previously.

MC252 crude oil was extracted into 75% and 90% EtOH when analyzed using the Fluorolog 3 (Figure 13). The spectrum using 75% EtOH showed high fluorescence between Ex320-380 and Em380-420. This was not in the range of HNP-like compounds but was in the range of the HPY standard (Figure 5A and 5B). The fluorescence shifted toward higher excitation and emission when crude oil was extracted into 90% EtOH, indicating extraction of higher MW PAHs. The effect of EtOH concentration on crude oil was compared to that on MVNJ fish oil. For MVNJ, the 75% EtOH appeared to extract Albumin (protein) with maximum fluorescence at Em340-360/Ex280. Alb-like fluorescence was not seen in crude oil. MVNJ also showed crude oil-like fluorescence with excitation ranging from 320-380nm and emission ranging from 380-420nm. Using 90% EtOH appeared to extract more crude oil-like compounds and less albumin-like compounds from MVNJ. MVNJ contour maps using 75% and 90% EtOH did not perfectly match those of MC252 crude oil. However, the MVNJ fish oil's fluorescence shifted up and to the left, simulating the effect seen with MC252 crude oil and indicating that MVNJ menhaden were exposed to and had accumulated crude oil-like compounds in their body oil.

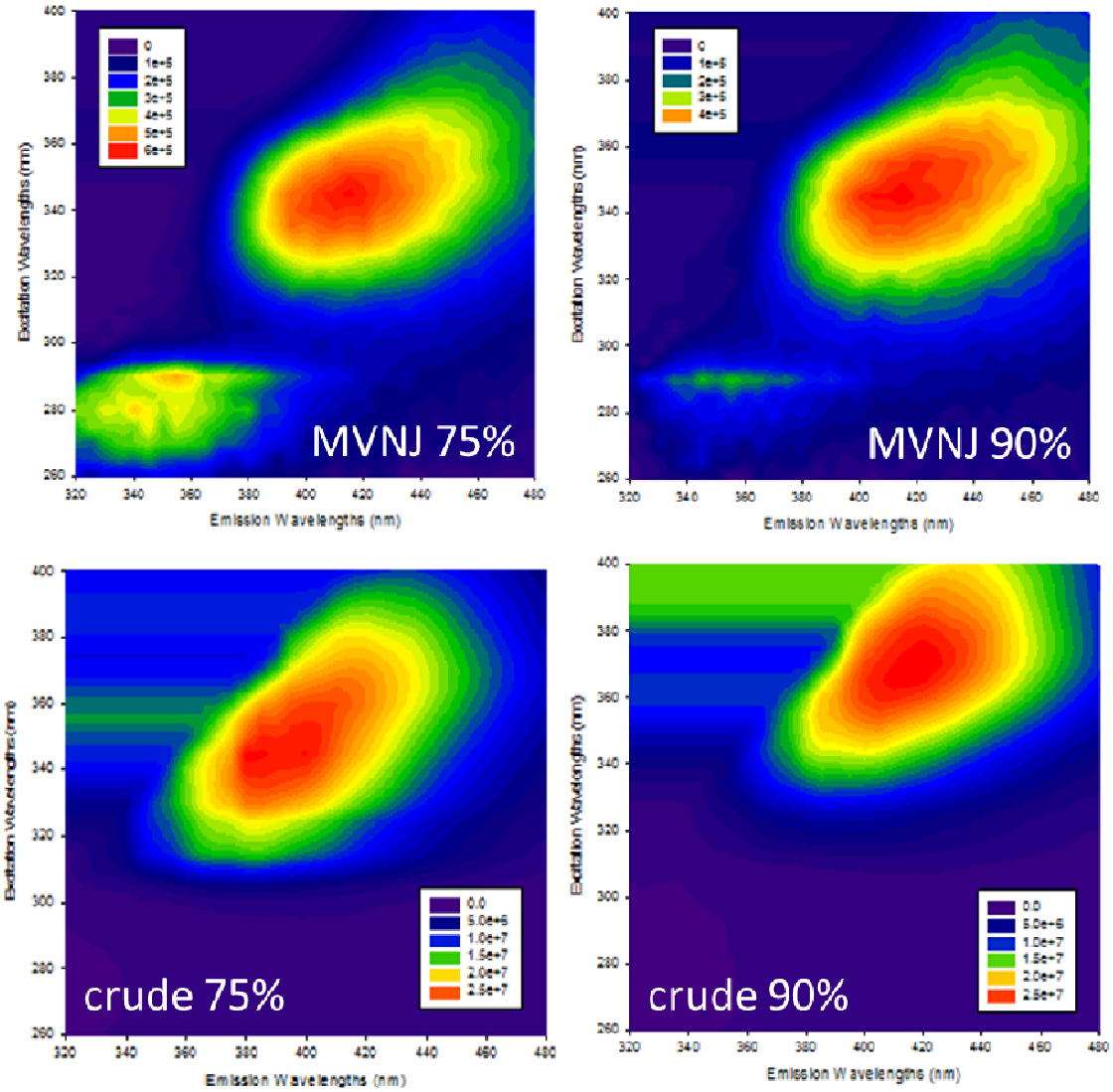


Figure 13. Contour map of 3D spectra comparing wild fish oil with crude oil. MVNJ was from menhaden collected at Delaware Bay, NJ in fall 2010. Crude oil (crude) was from the DWH riser pipe collected prior to the spill. Samples were extracted into either 75 or 90% crude oil. Spectra from MVNJ and crude oil were quite similar indicating the MVNJ menhaden were exposed to and had accumulated crude oil-like compounds into their body oil. Higher %EtOH shifted both MVNJ and crude oil spectra to higher excitation and emission wavelengths further indicating extraction of similar types of compounds from both samples, likely to be high MW PAHs.

Other wild caught menhaden fish oils were extracted into 75% EtOH and analyzed using the Fluorolog 3 (Figure 14). SHNJ and EPNJ1 were from menhaden collected in the Delaware Bay, NJ area in fall 2010. BBLA and VBLA were from menhaden collected at Barataria Bay and Vermillion Bay, LA in summer 2011. SHNJ, EPNJ1 and BBLA showed high MW PAH-like

compounds such that EPNJ1>BBLA>SHNJ. VBLA showed an albumin and/or HNP-like spectrum but no crude-oil like spectrum. EPNJ1 showed fluorescence at 360-460 nm emission, similar to the spectrum seen for MVNJ and MC252 crude oil (Figure 14 versus 13). MVNJ had the highest levels of crude oil-like fluorescence ($6e^{+5}$ CPS) among the fish oils analyzed by 3D spectra. Results showed that fish oils can contain different levels of fluorescent compounds proving that contamination can be detected in fish oil and that levels are site specific. In addition, data demonstrated that BBLA 2010 fish oil differs from BBLA 2011 fish oil suggesting that contamination in fish oil can vary by season. Also fluorescence of HPY-like PAHs were lower at VBLA than BBLA suggesting that MC252 from the DWH spill might have been detected at BBLA in summer 2011. These results supported the 2D spectra generated by fixing Em450 because SHNJ, EPNJ1 and BBLA showed high MW PAH-like compounds (Figure 14 versus 10b and 11b). However, results differ from the 2D spectra generated by fixing Em350 because the albumin and/or HNP-like compounds are not seen in the 3D spectrum. This may be because the high MW PAH-like compounds were masking the albumin and/or HNP-like compounds due to their high fluorescence intensity.

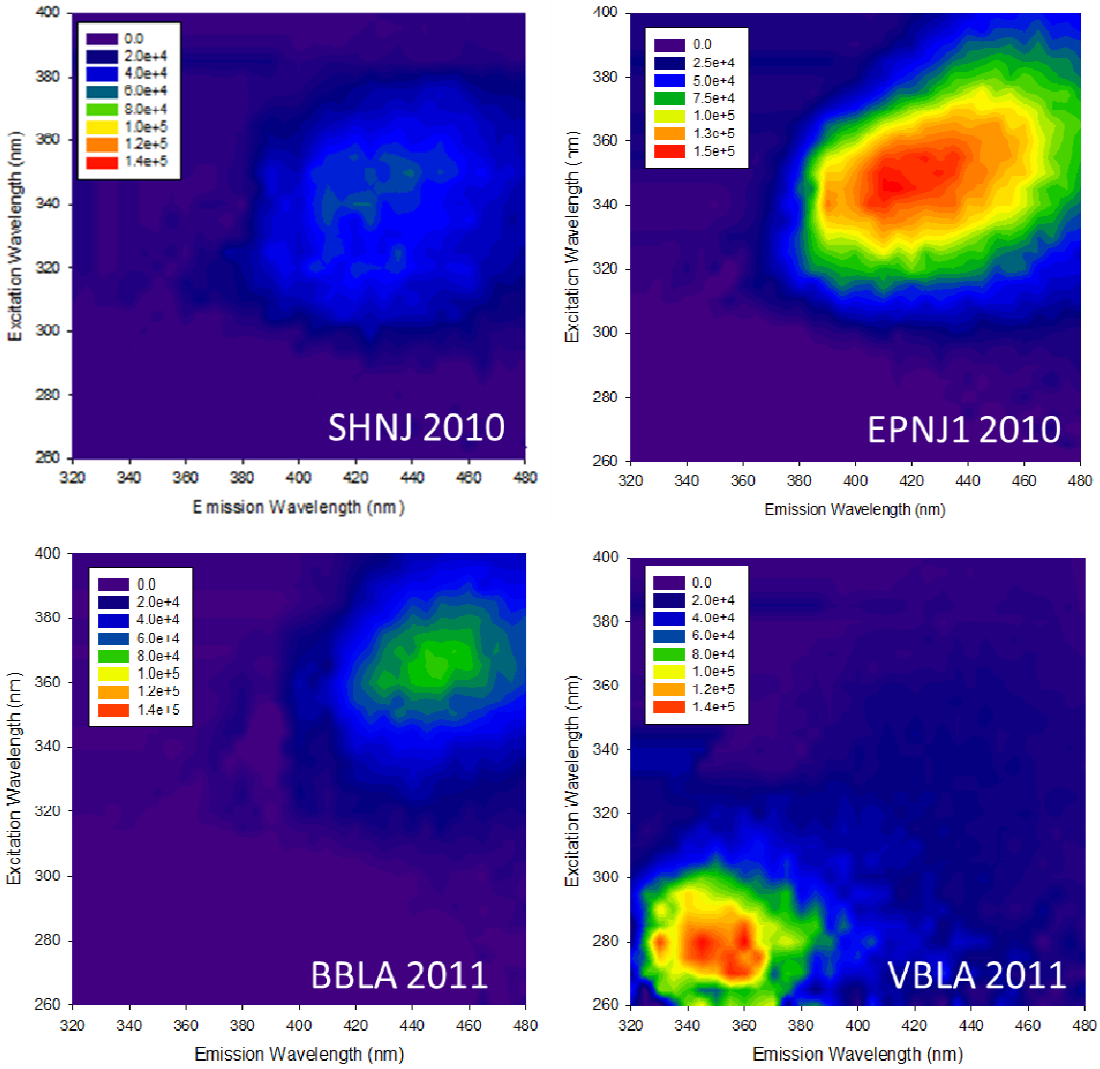


Figure 14. Contour maps of 3D fluorescence spectra for NJ and LA fish oil extracted into 75% EtOH. SHNJ and EPNJ1 were from menhaden collected at Delaware Bay, NJ in fall 2010. BBLA and VBLA were from menhaden collected at Barataria Bay and Vermillion Bay, LA in summer 2011. All samples were extracted into 75% EtOH. SHNJ, EPNJ1 and BBLA showed high MW PAH-like compounds such that EPNJ1>BBLA>SHNJ. VBLA showed an albumin and/or HNP-like spectrum. Results showed that fish oils can contain different levels of fluorescent compounds proving that contamination can be detected in fish oil and that levels are site specific.

Bile was obtained from killifish and compared to menhaden fish oils. The gall bladders were dissected out after being exposed to crude oil via a gavage method. The bile content was used for 3D SFS analysis. Results showed that bile from killifish experimentally exposed to crude oil in the laboratory contained PAHs and that bile from killifish not exposed was

distinctively different verifying that the gavage method was successful (Figure 15). Control bile showed fluorescent activity at Em350/Ex280 ($1.5e^{+6}$ CPS), indicating the presence of albumin. Crude oil exposed bile showed PAH-like fluorescence at Em350-410/Ex320-375, which was similar to the HPY standards (Figure 15 versus Figure 5a and b). These results supported the 2D spectra generated by fixing emission at either 350 or 450nm. The Em350 spectrum showed the presence of albumin (Ex280) and/or vitamin E (Ex290) in bile from unexposed fish (Control Bile) (Figure 15 versus 11a). The Em450 spectrum showed HPY-like fluorescence with a major peak at Em450/Ex350 in bile from exposed fish (Exposed Bile) (Figure 15 versus 11b). Data proved that crude oil and PAHs can be detected in bile of fish exposed to crude oil using SFS and that fluorescence from these compounds was similar to what was found in menhaden fish oil samples from MVNJ, SHNJ, EPNJ1, and BBLA 2011 (Figure 13 and 14). However, these fluorescent compounds were not found in the commercial fish oils tested.

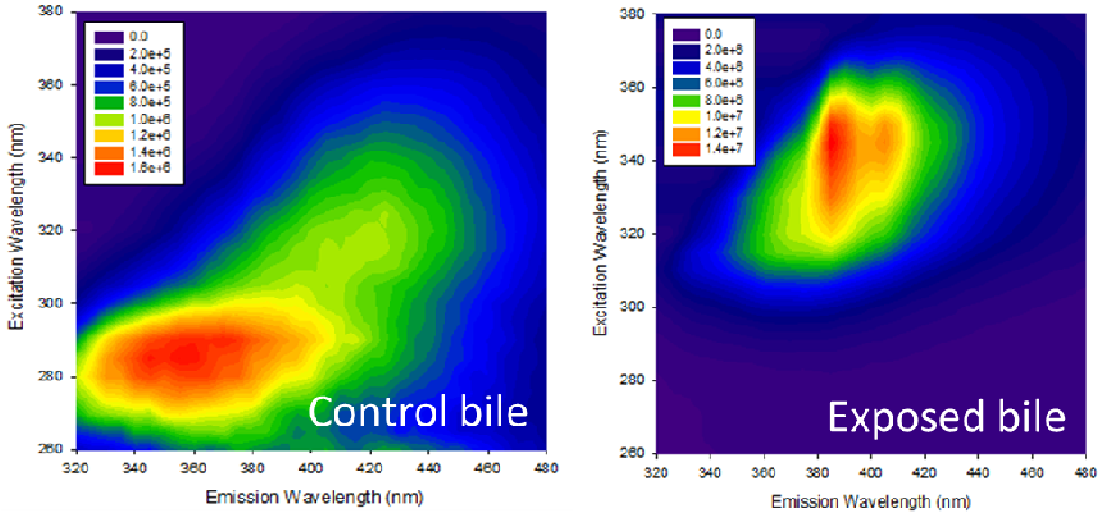


Figure 15. Contour map of 3D spectra comparing bile from control and crude oil exposed killifish. Samples were extracted into 75% EtOH. Bile was collected from killifish 3 days after exposure to crude oil collected from the DWH riser pipe prior to the spill. The spectrum from control (Control bile) shows fluorescence of albumin at 280ex/350em and possibly some PAH-like compounds accumulated from its environment prior to capture. The spectrum from exposed killifish (Exposed bile) shows a fluorescence spectrum similar to that for HPY at EX340/Em380. Data proves that we could detect the presence of PAHs from bile of fish actually exposed to crude oil.

Vitamins as a Confounding Factor when Detecting PAHs using SFS

Vitamins A and E and albumin may be detected instead of PAHs when biomonitoring oil spills using fluorescence. In particular, vitamin E and albumin may be detected at the fixed wavelengths used to measure HNP-like compounds. The ability of vitamins and albumin to interfere with PAH detection was studied by obtaining fixed wavelengths as well as EtOH concentrations for extraction from the literature (Table 6). According to the literature, researchers have used an Em335/Ex290 setting to detect HNP extracted in 48% EtOH (Kreitsberg, et al, 2010). Our results showed that HNP (491 RFU) was detected when using Em335/Ex290 but so was vitamin E (281 RFU) and albumin (601 RFU) in 48% EtOH. In addition, 75% EtOH provided higher fluorescence intensity for vitamin E (581 RFU) and albumin (763 RFU) when compared to HNP (467 RFU). All PAH concentrations were analyzed at 100 ng/ml, vitamin E was analyzed at 5,000 ng/ml, and albumin was analyzed at 30,000 ng/ml. Therefore, the RFU values generated by the Spectramax M5 cannot be directly compared. Interestingly, vitamin A did not fluoresce well at any settings used for biomonitoring PAHs. Albumin was detected at wavelengths used for 2-3 ring PAHs and could confound biomonitoring, especially when monitoring PAHs in tissue.

Table 6. Comparison of PAH standards and vitamins A & E at fluorescence wavelengths used for biomonitoring. Fluorescence intensity (RFU) is shown at the Ex/Em for compounds extracted into 48 or 75% EtOH. Biomonitoring wavelengths are shown in gray boxes. Note that Alb can fluoresce using all biomonitoring wavelengths . Vit A = vitamin A, Vit E = vitamin E, HNP= naphthol, HFL = hydroxyfluorene, HPY= hydroxypyrene, FAN = fluoranthene, BaP = benzo(a)pyrene. Vit E = 5000 ng/ml, Vit A= 25,000 ng/ml, PAHs = 100 ng/ml, Alb = Albumin = 30,000 ng/ml.

		RFU values						
%ETOH	Ex/Em	NPH	PHE	PYR	BaP	Vit A	Vit E	Alb
48	290/335	491	2	5	8	0	281	601
48	260/380	1154	5483	9374	686	0	8	357
48	341/383	81	344	20497	289	0	5	31
48	380/430	1	2	1536	10043	2	1	11
75	290/335	467	0	7	14	0	581	763
75	260/380	1230	7053	7824	682	0	14	395
75	341/383	112	445	17931	289	0	5	63
75	380/430	0	0	1451	9698	3	0	15

Discussion

The average American consumes between 1µg-5 µg a day of carcinogenic PAHs from unprocessed grains and cooked meats (Menzie et al, 1992). For this project, fish oil was obtained from menhaden species found in North America and from commercial supplements in order to investigate fish oil as a potential source of PAHs. Fish oil supplements can help maintain the elasticity of artery walls, decrease the risk of blood clotting, reduce blood pressure and stabilize heart rhythm- particularly decreasing the risk of CVD (Uauy-Dagach et al, 1996 and Connor et al, 2000). Fish oil tablets are made from oily fish like menhaden. Oil spills such as the 2010 DWH incident in the Gulf of Mexico, may contaminate oily fish with PAHs and thereby endanger marine fisheries as well as humans taking fish oil supplements for their heart health.

For this project PAHs in fish oil were detected using SFS. One method involved holding emission constant and scanning for excitation: this generated 2D spectra. Another method involved scanning excitation at multiple emissions which generated a 3D heat map. Testing of multiple PAH standards showed that low MW PAHs could be distinguished from high MW PAHs in 2D scans (Figures 3a and 3b). The 2D data supported the excitation scans for PAH identification because the numbers and pattern of the excitation peaks best distinguished between PAH standards. SFS is a fast and simple procedure for PAH detection (Albani et al, 2007). Fluorescence 2D spectral data are usually plotted as emission spectra and are dependent upon chemical structure of the compound of interest and the solvent in which the fluorescent compound is dissolved (Lakowicz et al, 1983). However, our 2D data was plotted as an absorption spectrum because it represents the excited vibrational state of the compound. This approach takes advantage of each compound having their own unique individual vibrational energy level and allows the spectra to show their different structures. The vibrational structures seen in the absorption and emission spectra are similar only because electronic excitation does not greatly alter the spacing of the vibrational energy levels (Albani et al, 2007). The peak seen in the absorption spectra corresponds to the electronic transition line, and the rest of the spectrum is formed by a series of lines that correspond to rotational and vibrational transition as shown in our data (Albani et al, 2007). Therefore, scanning for excitation would make a much more characteristic spectrum for all the PAHs under investigation as opposed to scanning for emission. PAH standards were also tested in 3D scans (Figure 5). The 3D spectra clearly distinguished between HPY and vitamin A; however, vitamin E, albumin, and HNP were somewhat difficult to distinguish due to overlying excitation and emission wavelengths. Detection and identification of PAH-like compounds in fish oil was found to depend on solvent interactions and the presence of

biological compounds naturally found in fish oils. Therefore, extensive method development was needed to verify the use of SFS.

Solvent Interaction

Results showed that 75% EtOH was the best concentration for extracting PAHs from fish oil. It allowed sufficient fluorescence intensity for detecting both HMW and LMW PAHs (Table 3). Comparing the percent recoveries in DB fish oil for the various standards indicated that the use of 75% EtOH could extract both hydrophilic and hydrophobic with at least 30% recovery for all compounds including HNP, Vit A, HPY, and albumin. Results for Vit E indicated very low percent recovery (4%) at 75% EtOH and higher recovery at 100% EtOH (12%). This indicated that Vit E is more hydrophobic and likes non-polar solvents. However, SFS spectra showed that concentrations of Vit E in fish oil samples were high enough for detection even with these low percent recoveries.

Interestingly, different EtOH concentrations affected the intensity and/or position of fluorescent peaks of compounds like albumin, Vit E, Vit A and low or high MW PAHs in both 2D and 3D spectra. For example, the 3D scan demonstrated that NM fish oil showed a higher intensity of vitamin E-like compounds at 90% EtOH, 5E6 cps/microamps, compared to 75% EtOH, 2E6 cps/microamp (Figure 12). This indicated that fluorescent compounds can be detected based on their hydrophobicity. The influence of solvent hydrophobicity was tested by spiking DB and NB fish oil with standards and extracting them into different concentrations of EtOH. Results of 2D scans showed that more low MW PAHs (HNP-like PAHs) were extracted at low EtOH concentrations (50%) while high MW PAHs (HPY-like PAHs) were extracted at higher ones (100%). For example, the 50% EtOH provided 35% recovery and 100% EtOH provided 2% recovery for HNP extraction; while the 50% EtOH provided 22.8% recovery and

100% EtOH provided 89.1% for HPY (Table 4). This indicated that high MW PAHs could be better detected in a higher EtOH concentration while low MW PAHs could be better detected using a lower EtOH concentration. Vit E or Vit A extracted in 100% EtOH produced a fluorescence peak 10xs higher than a 50% EtOH concentration extraction (Figures 6a and 6b). Nevertheless, 75% EtOH provided sufficient fluorescent intensity as well as sufficient percent recovery for all standards.

Moreover, the percent EtOH influenced the variety of fluorescent compound that were extracted from fish oil. Data showed that NB and DB fish oil showed different fluorescence spectra when extracted into different EtOH concentrations (Figures 7a, 7b, 8a and 8b). For example, the 3D scan showed that NB fish oil had primarily vitamin E-like compounds (Em350/Ex290) at 90% EtOH and HNP-like (Em350/Ex270) PAH at 75% EtOH. Research has indicated that fluorescence spectra can be dependent on the detailed chemical structure of the fluorophore as well as the type of solvents (Lakowicz et al, 1983). Therefore, this supported the finding that the solvent can alter what type of compound is extracted from fish oil.

Another explanation for observed changes in fluorescence spectra could have been the suppression or enhancement of fluorescence itself. Suppression may have occurred due to the combination of fluorescent compounds in the fish oil sample forming a charge transfer complex, called excimer formation, which can be referred to as exciplexes (Valeur et al, 2013). Exciplexes are known to be dimer compounds that branch together and provide a characteristic spectrum as best exemplified by NB 2D scans because it loses the Em350/Ex270 and provides a Em350/Ex310 peak (Figure 8a), and the 3D scan shows NB losing the Ex270 and shifting up to Ex300 (Figure 12). This indicated that the fish oil may contain more than one fluorescent compounds sticking together forming dimers; which then alter the fluorescent spectra. Other

interferences in detection of PAH-like compounds may have been fluorescent compounds in the solvent and solutes suspended in samples causing stray light scattering called Raman lines (Valeur et al, 2013). These interferences were subtracted out of the spectra presented, but they may contribute suppression or enhancement of fluorescent intensity.

Fish Oil

PAH-like compounds were detected in commercial and wild fish oils shown by 2D and 3D scans. PAH standards demonstrated characteristic excitation peaks that were often similar to those for fish oil spectra. For example, 2D spectra of HNP provided two peaks: one major peak at Em350/Ex270 and a minor one at Em350/Ex320. MVNJ fish oil had one major peak in the same area ranging from Em350/Ex270 to Em350/Ex280. The 2D spectrum of HPY provided two peaks: one at Em450/Ex270 and another at Em450/Ex340. JRVA and MVNJ fish oil both had one major peak in the same area as the second excitation peak of HPY (Em450/Ex340). The absence of the first peak could have been due to excimer formation. Fish oils had major peaks that were similar to those of standards; however, the peaks were not unique enough to detect specific types of PAHs.

Excimer formation appeared to account for the quenching or suppressing of fluorescence peaks in fish oil and combinations of some standards. PAHs such as HNP and HPY can form excimers with other compounds in solution. For example, HNP absorbed at 270 nm (short wavelength) and 320 nm (long wavelength) and emitted light at 350 nm. HPY absorbed light at 270 and 340 nm and emitted light at 450 nm. An excimer could have been formed when one monomer of HNP bound with one monomer of HPY. This excimer had a different vibrational structure that reduced the first excitation peak and enhanced the second resulting in an alter spectrum for the combination of fluorescent compounds (Valeur et al, 2013). Excimer formation

may have explained why the 2D and 3D spectra of some fish oils only showed one major area of fluorescence (Figures 13 and 14) instead of two or more as seen for HPY alone (Figure 7b). For example, the 3D spectra of HPY combined with vitamin A showed that HPY can lose fluorescence intensity of its first peak at Em410/Ex270; however, when combined with albumin instead, HPY loses fluorescence intensity of its second peak at Em410/Ex340 (Figure 5). Therefore, the presence of vitamin A and albumin standards affected the spectrum of HPY. Fish oil also affected the spectrum of HPY. 2D scans of HPY alone showed two peaks of similar fluorescence intensity, Em450/Ex270 and Em450/Ex340. However, when spiked into NB fish oil and extracted into 75% EtOH, only one major peak at Em450/Em340 was found (Figure 7b). Therefore, the fish oil components or the matrix itself affected the spectra and where the optimal detection of PAH-like compounds in fish oils would be found.

The spectra or intensity levels of fluorescent compounds were affected by the method of detection. For example, 2D spectra of some fish oils; such as GNC, BBLA 2011, SHNJ, and EPNJ2 had a peak at Em450/Ex330 which was similar to that of vitamin A (Figures 9b, 10b, and 11b). However, the 3D spectra only showed vitamin A-like compounds in Lovaza but not any other fish oils (Figure 12). The peak seen for some fish oils at Em350/Ex290; such as, NM, SV, NB, JRVA, and BBLA 2010 appeared to match that of vitamin E (Figures 9a, 10a and 11a). In this case, the 3D spectra did suggest vitamin E-like compounds in NB, NM, and LV but not in any wild menhaden fish oils (Figures 12 and 14). In addition, vitamin E spiked with HNP showed an Em350/Ex280 peak (Figure 4a). This suggested that the Em350/Ex280 peak detected in wild and commercial fish oils was a mixture of HNP-like PAHs and vitamin E. However, 3D spectra suggested that the Em350/Ex280 may have been albumin instead (Figures 5 and 9a). The difference between the two types of spectra might have been due to contour waves. These waves

resulted from the broad nature of fluorescence peaks such that the highest fluorescence occurred at the optimal Em/Ex of a compound but was also detected at lower intensities away from the peak. This was seen in 3D scans as the red color associated with maximum fluorescence transitioned to light blue away from the peak fluorescence (Figures 5, 12-15). Comparison of 2D and 3D spectra indicated that 2D spectra were affected more than the 3D spectra by contour waves. The 3D spectra were better at distinguishing between the types of compounds present and detected compounds not detected by the 2D spectra.

Comparison of commercial fish oil spectra showed considerable differences between supplements. Commercial fish oil seemed to contain mostly vitamin E. However, Lovaza appeared to also contain a form of vitamin A. In 2D scans, Vitamin A was detected at Em450/Ex330 (Figure 6b); while in 3D scans it was detected between Em450-480/Ex310-340 (Figure 5). The 2D scan of Lovaza had two peaks, one at Em450/Ex320 and one at Em450/Ex350 (Figure 9b). This was in its 3D spectra and in the same range of vitamin A (Figure 12). In addition, the vitamin-A like peak of Lovaza was better extracted by 90% than 75% EtOH: another characteristic of vitamin A. Vitamin A was not one of the ingredients listed by the manufacturer. Nature's Bounty appeared to contain fluorescent compounds other than vitamins. It had a major peak at Em450/Ex270 and a minor peak at Em450/Ex350 (Figure 9b). This combination of peaks was similar to that of FAN using 2D spectra (Figure 3b). However, 3D spectra did not confirm the presence of HMW PAHs (Figure 12). NB also had a shoulder at Em350/Ex270 using 2D scans, which was indicative of HNP-like PAHs (Figure 9a). This finding was supported by 3D scans (Figure 12). Taken together, the data suggested that NB fish oil may be contaminated with LMW PAHs. Other unknown fluorescent compounds may have accounted for the fluorescent spectra observed.

Results indicated that SFS could detect different levels of PAH-like compounds in wild fish collected from different locations. For example, fish collected from JRVA contained higher levels of HPY-like PAHs than those collected from the Delaware Bay, NJ area (Figures 10b and 11b). Data demonstrated higher levels of PAH-like compounds in MVNJ and EPNJ1 than BBLA 2011 fish oil. This indicated that NJ fish oil had contaminants from pyrogenic sources (combustion of oil-based products) as opposed to petrogenic sources (petroleum released), because there have been no recent large oil spills in NJ. Menhaden collected from BBLA in fall 2010 contained lower levels of PAHs than menhaden collected the following summer, BBLA 2011. The low PAH levels in fall 2010 were surprising as this collection period was closer to the DWH event than the summer 2011 collection. The reason for the low levels in fall 2010 was unknown. However, menhaden collected from BBLA in 2011 showed crude oil-like spectra, while those from VBLA did not (Figures 13 and 14). This was in keeping with heavy oiling of BBLA and not VBLA during the DWH spill. Overall, results indicated that fish oil could be used to detect PAH exposure and compare one site to another.

The 3D spectra for some fish oils resembled that for DWH crude oil. The spectra for MVNJ and EPNJ1 showed a region of fluorescence that was very similar to the DWH crude oil spectrum (Figures 13 and 14). Interestingly, the fluorescent spectra of DWH crude oil and MVNJ shifted up and to the right when the EtOH concentration was increased from 75 to 90% (Figure 14). This indicated that MNJ fish oil and DWH crude oil contained similar fluorescent compounds. The spectrum for the BBLA 2011 fish oil was also similar to the DWH crude oil spectrum; however, the fluorescent compounds extracted from the BBLA 2011 fish oil were shifted toward higher Ex/Em wavelengths. Based on the results of solvent hydrophobicity experiments, this indicated that BBLA2011 had relatively more HMW PAHs than DWH crude

oil. This could have been due to exposure to aged crude oil at the time of collection coupled with bio-elimination of lower MW PAHs. Research has shown that PAHs are rapidly bio-eliminated by organisms after uptake (Cailleaud et al, 2009). The VBLA fish oil sample collected in the summer of 2011 did not show the crude oil signature seen in BBLA 2011. This indicated that VBLA served as a negative control. The data supported that PAHs could be extracted from fish oil and that crude oil could be a source of the PAHs detected in the wild fish oils tested.

High performance liquid chromatography has detected PAHs in edible oil, such as fish oil, and the results revealed that fish oil contained a total of 0.051 µg/g of low MW PAHs, and 0.002 µg/g of high MW PAHs (Wang et al, 2013). The concentrations of PAHs-like compounds detected in menhaden fish oil as well as commercial fish oil were determined by using a standard curve and dividing by the average weight of the fish oils (Table 5). The SFS data showed 100 times higher concentrations in the commercial and wild fish oils compared to Wang et al (2013). The range for LMW PAHs was 0.11-48.85 µg/g with NB fish oil containing the highest concentration. The range of HMW PAHs was 0.16-80.2 µg/g of HMW PAHs with GNC containing the highest concentration. Wang et al did not provide the fish oil manufacturer; therefore, our fish oil cannot directly be compared to theirs. Our SFS data probably represented other fluorescent compounds not detected by the HPLC method including Vit E-like compounds and PAH metabolites. Data using fixed wavelengths suggested that commercial fish oils may not be safe to consume, specifically NB and GNC because it has been reported that the maximum levels for PAHs in certain foods like oils and fats are 0.005 µg/g (Motarjemi et al, 2014). However, observation of both 2D and 3D spectra of GNC showed that the major peak was not located in the area of HMW PAH standards or crude oil. The fluorescence intensity used to calculate PAH levels was actually due to contour waves, leading to misleading results. This

demonstrated that 2D and 3D spectra were better at identifying fish oil components than fixed wavelengths. The data also indicated very high contents of Vit E, Vit A, and albumin (Table 5). High concentrations of these compounds could have artificially increased the LWM PAH-like compounds in NB. However, 3D spectra did indicate the presence of something other than vitamin E in NB. Overall, 3D spectra were superior for identifying fish oil components.

Bile Data

Bioaccumulation of fluorescent compounds following exposure to DWH crude oil was investigated by analyzing bile from killifish using the SFS procedure developed for fish oil. Results indicated that the exposure group had higher concentrations of PAH-like compounds than the control group demonstrating that the crude oil had been absorbed through the gut (Figures 11a, 11b, and 15). The 2D and 3D spectra for exposed bile was very similar to that seen for NJ and BBLA 2011 fish oils indicating that the fluorescent spectra in wild fish oils could have been due to PAH exposures (Figures 10a, 10b, 11a, 11b, 14, and 15). Current research has found elevated levels of PAH metabolites in fish bile from the Gulf of Mexico (Murawski et al, 2014). Overall, data proved that PAH-like compounds can be detected in bile of fish exposed to crude oil using SFS and that fluorescence from these compounds was similar to menhaden fish oil samples from MVNJ, SHNJ, EPNJ1, and BBLA 2011.

Confounding Factors

SFS data showed that vitamins could be confounding factors when detecting PAHs. Biological molecules can contain natural occurring or intrinsic fluorophores (Lakowicz et al, 1983). For example, amino acids such as tryptophan and tyrosine can absorb light at 280 nm and emit light at 350 nm resulting in fluorescence of proteins. Vitamin A can fluoresce at Em450/Ex328, and vitamin E can fluoresce at Em350/Ex290. Commercial fish oils can naturally

contain vitamin E (McNight, 2014 and Young, 1986), but it can also be added in order to preserve the n-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Traber and Wander, 1999). Commercial fish oils can also contain vitamin A (retinol) as an additive, which is sometimes indicated on the label (McShane, 2014). This increases the fluorescence intensity of vitamin E and vitamin A compounds that are detected in commercial and wild caught menhaden fish oils. Fish oils can contain 25-60 µg/g of vitamin E and 2-7 µg/g of vitamin A (Young, 1986). The SFS data indicates that commercial and wild caught menhaden fish oils can contain concentrations of Vit A-like compounds between 4-6365 µg/g of fish oil and Vit E-like compounds between 0.3-1904 µg/g of fish oil (Table 5). A food Fluor library provides 3D contour maps for vitamin and protein standards that are similar to ours (Christensen, 2005). Analyses of fixed excitation and emission wavelengths showed that wavelengths that detect HNP-like PAHs may detect vitamin E and/or albumin (Table 6). According to our data, the use of 3D as opposed to 2D and fixed wavelengths may improve biomonitoring of PAHs.

Conclusion:

Methods were developed for detecting PAHs in fish oil. Results demonstrated that PAH-like compounds can be detected in fish oil using fluorescence. Comparison of fish oil 2D spectra to 3D spectra showed the presence of similar compounds; however, 3D spectra better identified specific types of compounds. PAH-like substances were quantified in commercial and wild fish oils at the ppm level. Some menhaden fish oil from Delaware Bay, NJ and James River, VA had higher PAH-like compounds than fish from Barataria Bay, LA, where the DWH oil spill occurred. However, levels in fish oil from Vermillion Bay, LA were lower suggesting that the contamination seen at Barataria Bay was from the spill. Vitamin A and E peaks were present in

some wild fish oils and all commercial fish oil products. Some commercial fish oils had unexpected fluorescent compounds such as vitamin A in Lovaza and HNP-like PAHs in Nature's Bounty. Many researchers have used SFS technologies for detecting PAHs. Our data indicated that levels of HNP-like PAHs reported in their studies could actually reflect concentration of vitamin E or albumin instead. Future work should provide a better method for differentiating between vitamins and PAHs. Overall, menhaden fish oil could be useful for monitoring oil spill contaminants; however, it is not yet possible to distinguish between PAHs accumulated from crude oil spills and those from burning of fossil fuels.

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