USE OF DIETARY POLYPHENOLS TO STABILIZE POLYUNSATURATED FATTY ACIDS IN FOOD

By

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Ilya Raskin

And approved by

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## ABSTRACT OF THE THESIS

**Use of Dietary Polyphenols to Stabilize Polyunsaturated Fatty Acids in Food**

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Omega-3 fatty acids (n-3 PUFAs) are a subset of polyunsaturated fatty acids found primarily in marine food sources as eicosapentaenoic acid (EA) and docosahexaenoic acid (DHA), and in plants as α- linolenic acid (ALA). n-3 PUFAs are essential nutrients demonstrated to have positive effects on inflammation, coagulation, lipid profiles, cardiovascular disease, cognitive impairment, and psychological disorders. Western populations historically under-consume whole food sources of n-3 PUFAs making n-3 PUFAs supplementation popular. The most common form of n-3 PUFAs supplementation, fish oil, is prone to oxidation which limits shelf-life and palatability. Polyphenols have been demonstrated to exert protective effects on marine derived n-3 PUFAs in solution. However, liquid states limit supplement storage and delivery options. A co-dried matrix of whey protein isolate (WPI), triglyceride oil (TG oil), and grape polyphenol extract (GSP) could provide a dry, shelf-stable delivery system for n-3 PUFAs supplements. Stability projects showed an improved recovery of EPA and DHA methyl esters from the co-dried matrix compared to the free TG oil alone, however a

greater recovery of n-3 PUFAs was seen with an unprotected WPI and TG oil matrix when compared to a GSP- WPI-TG oil matrix demonstrating a protective effect of protein on n-3 PUFAs independent of polyphenols.

## Acknowledgements

While writing the acknowledgement is generally left to the end of the dissertation process I have written this in the summer months of 2018 for I am already keenly aware as towards whom I shall express my sincerest gratitude.

First, to my advisor, Dr. Ilya Raskin, for your continued mentorship and patience during these long, arduous years; it hasn't been an easy road, but I look forward to sharing a solid 'high-five' at the end. To my committee members (both present and former), Drs. Judith Storch, Tracy Anthony, and Dawn Brasemle, for their critical review of my research and encouragement of my professional endeavors. To Dr. David Ribnicky for guidance, always knowing how to fix the GC-MS, and making delicious pasta dishes. To the entity that is the Raskin lab. We have grown and changed together over these many years- thank you for your collective support. Finally to my family, friends, and doggies for without your support and hugs I would have never made it to the end. But mostly, 'thank you' to my husband, Marwan, for Lord knows he deserves it the most.

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## List of Abbreviations

α-linolenic acid (ALA)

Adequate Intake (AI)

Arachidonic acid (AA)

Butylated hydroxyanisole (BHA)

Butylated hydroxytoluene (BHT)

Cardiovascular disease (CVD)

Coronary heart disease (CHD)

Docosahexaenoic acid (DHA)

Eicosapentaenoic acid (EPA)

Grape polyphenol extract (GSP)

Grape seed extract (GSE)

High density lipoproteins (HDL)

Low density lipoproteins (LDL)

Omega-3 fatty acids (n-3 PUFAs)

Polyunsaturated fatty acids (PUFAs)

Recommended Dietary Allowance (RDA)

Triglyceride oil (TG oil)

Very low density lipoproteins (VLDL)

Whey protein isolate (WPI)

# 1. Introduction and Literature Review

## Omega 3 fatty acids and health

Omega- 3 polyunsaturated fatty acids (n-3 PUFAs) are long chain PUFAs essential for human health. The three main n3-FAs are α-linolenic acid (ALA, 18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). ALA can be found in nuts, seeds, and vegetable oils specifically flax, walnut and soybean, while EPA and DHA are found only in marine food sources, specifically oily fish[1]. EPA and DHA cannot be synthesized *de novo,* they can be synthesized through the enzymatically elongation of ALA; however, hepatic conversion of ALA to EPA and DHA is slow and inefficient (see Fig 1.). This is partly because the rate-limiting enzyme Δ6-desaturase is active towards n-6 fatty acids as well as n-9 fatty acids, resulting in potential competition for enzyme activity between the families of fatty acids[4]. In vivo studies have shown that animals receiving ALA supplementation have lower systemic concentrations of EPA and DHA levels than animals fed the preformed version of either EPA or DHA. Similar clinical studies showed that patients given high doses of ALA have increased plasma levels of EPA upon ALA supplementation but no significant increase in DHA levels[2]. While results vary, stable isotope methods determined the average conversion of ALA to DHA being less than 1%[4].

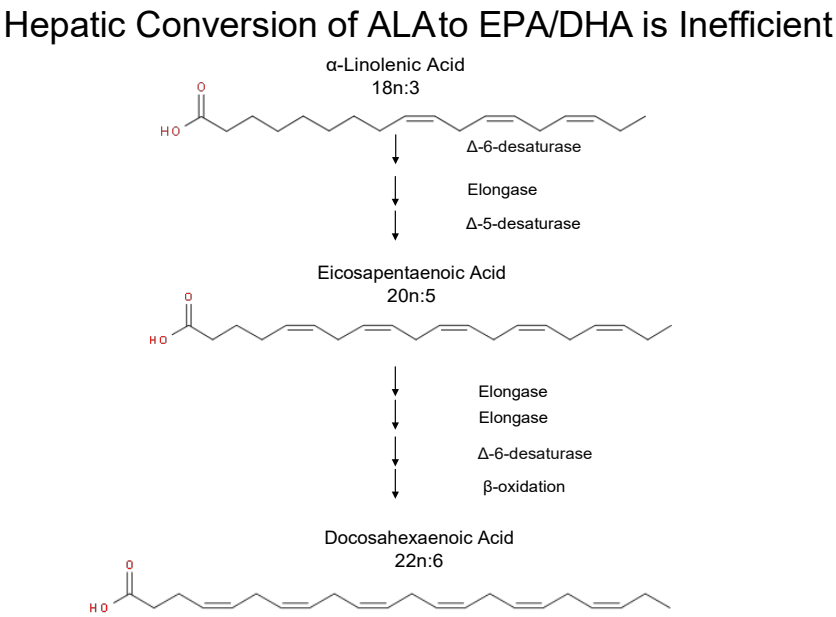



**Figure 1. Graphical representation of hepatic n-3PUFA conversion**

Cardiovascular disease (CVD) is defined by the World Health Organization as a group of disorders of the heart and blood vessels that includes coronary heart disease (CHD), cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, and deep vein thrombosis and pulmonary embolism. It is one of the leading causes of death and disability in North America[34, 38]. The most common acute clinical manifestations of CVD are myocardial infarction and stroke. In most cases the underlying cause of CVD is athlerosclerosis[35].

n-3 PUFAs, specifically EPA and DHA, have long been associated with improved cardiovascular health. This association gained attention since the 1978 observation that Greenland Inuit populations experience a low incidence of cardiovascular disease (CVD) events specifically myocardial infarction (MI), bleeding, low levels of low density lipoproteins (LDL) and very low density lipoproteins (VLDL), and high levels of high density lipoproteins (HDL)[3]. This was determined to be an effect of diet. Researchers

concluded that the high levels of EPA in the Inuit population did not induce platelet aggregation and that high levels of plasma EPA combined with low levels of arachidonic acid (AA, 20:4n-6) could lead to an antithrombotic state. These data along with studies like the Nurse Health Study[36], and the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI)-Prevenzione[36] demonstrated that a higher intake of fish or n-3 PUFA supplements resulted in a reduction of CHD mortality[36], non-fatal MI and stroke[37]. Additionally, n-3PUFAs from dietary sources and supplementation are associated with reduced inflammation[9, 21, 33], improved lipid profiles[22, 24], increased muscle protein synthesis[23], an attenuation of psychiatric disorder symptoms[5, 6, 7], delayed cognitive decline[8], and improved fetal and infantile cognitive and photoreceptor development[31, 32].

**Supplement Usage**

The use of nutritional supplements is a regular part of daily life in the western world with approximately 50% of US adults consuming some type of micronutrient supplement[25]. It is a common habit to begin multivitamin supplementation in early childhood and continue supplementation through adulthood. The rationale behind the use of supplements is simple: to promote general health, improve nutritional status, and to potentially prevent onset or further development of disease[25-29].

The third most common dietary supplement taken in the US, after multivitamins and calcium, is an n-3 PUFA or fish oil supplement[28, 30] and the prevalence of n-3PUFA supplementation is increasing[25]. Due historically low fish consumption in the west, fish oil supplementation is a recommended means to increase n-3 PUFAs in the diet. While

there is no Recommended Dietary Allowance (RDA), the American Heart Association recommends an Adequate Intake (AI) of 1.1 – 1.6 g/day of total n-3 PUFAs for adults[39].

Due the large number of double bonds within the fatty acid carbon chain, n-3 PUFAs are prone to oxidation. This occurs when a hydrogen atom is removed from a carbon-carbon double bond in an unsaturated fatty acid. In order to stabilize this alkyl radical, the fatty acid will transform from a *cis* to *trans* configuration. The radical can then react with molecular oxygen to form a high-energy peroxyl radical which can then remove a hydrogen atom from an adjacent double bond. Lipid oxidation results in the production of hydroperoxides and secondary oxidation products. These peroxides create a discoloring and fishy odor that is offensive to some[14, 17].

Currently fish oil supplements are sold as a gel-encapsulated product with an added antioxidant, usually α-tocopherol, and sometimes an added flavoring such as lemon oil. While fish oils are a popular supplement, they pose issues with consumer deliverability. Even with encapsulation, shelf life of the oil is limited due to rapid oxidation rates[30, 32]. The addition of other natural antioxidants may offer protection against rancidity, promoting shelf-life and potentially supplement efficacy.

**Polyphenols and fatty acids protectants**

Extracted polyphenols from grape and green tea are shown to protect omega-3 PUFAs from oxidation in a liquid state. Green tea catechins can act as antioxidants by donating a hydrogen atom, accepting free radicals, or by chelating metals[16]. Oxidative stability is demonstrated in ground fish meal and marine-derived oils when treated with green tea extract compared to other commonly used antioxidants[17, 18]. Resveratrol from wine is

more active than catechins in preventing LDL peroxidation[19] and extracted grape pomace polyphenols are found to slow the rate of oxidation in fish containing lipids, including bulk oils and frozen fish (mackerel) muscle[20].

**Development of protein-polyphenol complex**

Polyphenols, phytochemicals found in plants, are a series of organic compounds classified by multiple phenol structures and are identified as anti-inflammatory, antioxidant, anti-carcinogenic and anti-mutagenic agents[10]. They are secondary metabolites not involved in growth and energy metabolism in the body[11] and are the largest source of dietary antioxidants[12]. Polyphenols display an affinity for binding to both soluble and insoluble protein complexes specifically long chain peptides rich in proline residues. Polyphenols are prone to rapid degradation when isolated from polysaccharides in the whole food source as is done in commercial processing. The protein-polyphenol complex utilized in this thesis is based upon a previously published model used to stabilize extracted fruit polyphenols[13]. Once sorbed to the protein matrix, the polyphenols are photo- and thermo-stable.

The primary objective of this project was to determine an alternative method of supplemental n-3FA delivery that promotes stability and improves shelf-life. It was hypothesized that the incorporation of triglyceride oil into the polyphenol-protein complex would provide an improved stabilization of the fatty acids compared to free triglyceride oil. In order to test this hypothesis, the project design consisted of three individual projects. Project 1, labeled as oxidative conditions, and Project 2, labeled as reductive conditions, were designed to test shelf life stability under post- and pre-consumer purchase storage conditions while Project 3 was designed to test these methods

in a potential commercial application. The project outline is described in the figure below.

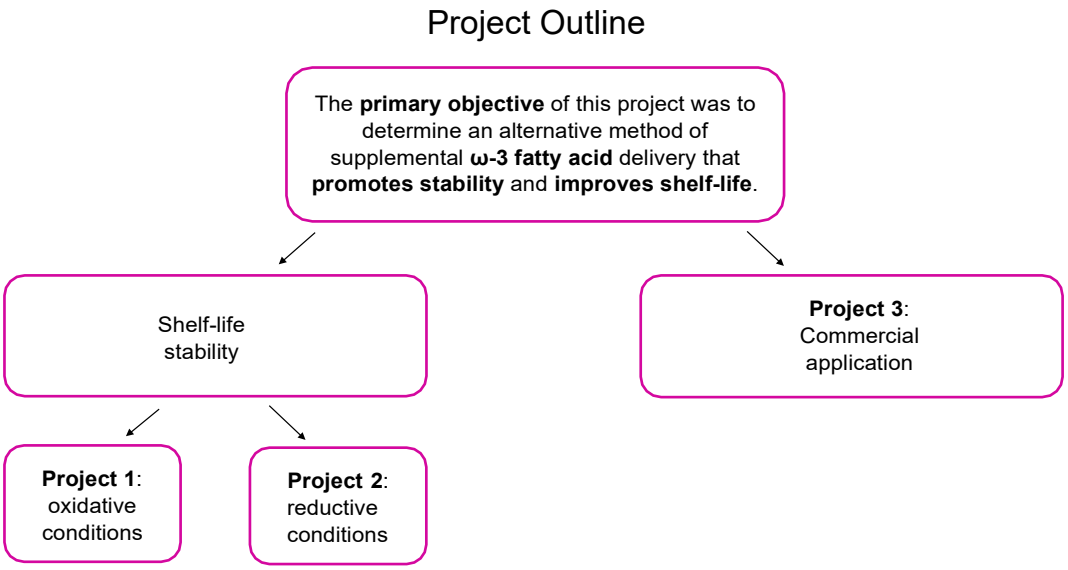



**Figure 2. Schematic representation of thesis project outline**

## 2. Materials and Methods

The thesis was conducted in three separate projects.

### Project 1: Recovery of n-3 FAs from storage under oxidative conditions

**Materials**. DHA standard cis-4,7,10,13,16,19-Docosahexanoic acid (≥98%), EPA standard cis-5,8,11,14,17-Eicosapentaenoic acid (≥98%), hydrochloric acid (HPLC grade, 1N) and Folin & Ciocalteu's phenol reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hexane (HPLC grade 95% n-hexanes) was purchased from Thermo-Fisher Scientific (Hampton, NH, USA). Methanol (HPLC grade) was purchased from VWR International (Randor, PA, USA). GNC Pro Performance 100% WPI was

provided by GNC (Pittsburgh, PA, USA) and MEG-3 1812 TG oil provided by DSM (Heerlen, The Netherlands).

| | EPA | DHA | Total n-3 PUFAs |
|---|---|---|---|
| As TG Oil (mg/g) | 160 | 110 | 320 |
| As Free Fatty Acids (mg/g) | 150 | 110 | 320 |

**Table 1. Fatty acid profile of the DSM provided Meg-3 1812 oil as stated on the DSM Certificate of Analysis**

Grape seed extract (GSE) provided by Changsha Huir Biological – Tech Co. LTD (Changsha, China). Food dehydrator model 3900 was purchased from Excalibur Dehydrator (Sacramento, CA, USA). White Connaisseur brand coffee filters (8-12 cup basket) were purchased locally. Epica electric coffee and spice grinder was purchased from Amazon.com.

**Polyphenol protected triglyceride production**. Grape polyphenol extract (GSP) was made by mixing GSE with 25% aqueous ethanol (made with Millipore water) for 20 minutes at room temperature in a ratio of 1 g of GSE to 5 ml of solvent. The mixture was then centrifuged for 10 minutes at 3200 x g. The recovered supernatant and dried extract were then quantified for total polyphenol content using the Folin Ciocalteu method. Briefly, 100 ul of extract (diluted in water) was mixed with 100 ul 0.25N Folin Ciocalteu reagent and left to incubate at room temperature for 3 minutes before adding 100 ul of 1M sodium carbonate. The total mixture was left to incubate for 5 minutes at room temperature before adding 700 ul of Millipore water. The samples were read at 726 nm using Biotek Synergy HT spectrophotometer (Winooski, VT, USA).

TG oil dispersed in hexane (1:8 v:v ratio) was mixed with the GSP at room temperature. WPI was added to the GSP-TG oil mixture to achieve a dry weight of 25% total TG oil and either 5% or 15% total grape polyphenols. The slurry was stirred for 10 min at room temperature using a stir bar and plate, poured into coffee filters and dried for 48 hours at 40°C in the food dehydrator. The amount of WPI and GSP added to the mix to make a 5% or 15% total polyphenol final dry product was calculated based on the total polyphenol measurement of the GSP as well as the amount of non-polyphenol dry residue contributed by the extract. As the percentage of polyphenols in the complex increased, the volume of GSP extract used in the mix increased which also increased the amount of non-polyphenol dry residue contributed to the weight of the dry complex. Therefore the amount of protein isolate required to achieve a dried complex of 25% TG oil and either 5% or 15% total polyphenols decreases as polyphenol content increases. The amount of TG oil added to each complex remained constant for a final, dried complex of 25% TG oil based upon dry weight of the complex.

A TG oil-fortified WPI control was prepared in the same manner with the elimination of GSP and the replacement of 25% ethanol. A control WPI sample was prepared by mixing with 200 ml of hexane and 300 ml of 25% ethanol. Dried samples were ground to a fine powder.

**Experimental Design**. Equal amounts of each dried sample and free, liquid TG oil were placed into cell culture multiwell plates, covered and taped shut, wrapped in aluminum foil to protect from light, and placed at 4°C, room temperature (approx. 20°C), and 37°C. For stability measurements, a 200 mg aliquot from three separate wells per individual dried complex and a 50 mg of TG oil was taken at each collection point producing a set

of triplicate measurements per dried complex or oil per collection point. Each well was sampled once at each collection point and no well was sampled more than once.

To elute the TG oil from the WPI-polyphenol complex, samples were vortexed in 1.6 ml of chloroform and methanol (1:1 v:v) for 30 seconds. Samples were then centrifuged at 3200 x g for 15 minutes and the eluted complex pellet was discarded. The solvent was removed from the eluted TG oil samples through low temperature speed-vac evaporation. Each eluted TG oil sample was hydrolyzed in 2 ml of methanol containing 5% HCl for 1 hour in a 90°C water bath. After removal from water bath, an equal volume of hexane (2 ml) was added to the methylated samples. Samples were vortexed for 5 seconds and centrifuged for 5 minutes at 3200 x g. The organic layer was recovered and stored at -20°C prior to GCMS quantification. DHA and EPA methyl esters were then quantified using GC-MS. Separate standard curves for DHA and EPA methyl esters were produced in the same manner using DHA and EPA standards (Sigma Chemicals, St. Louis, MO).

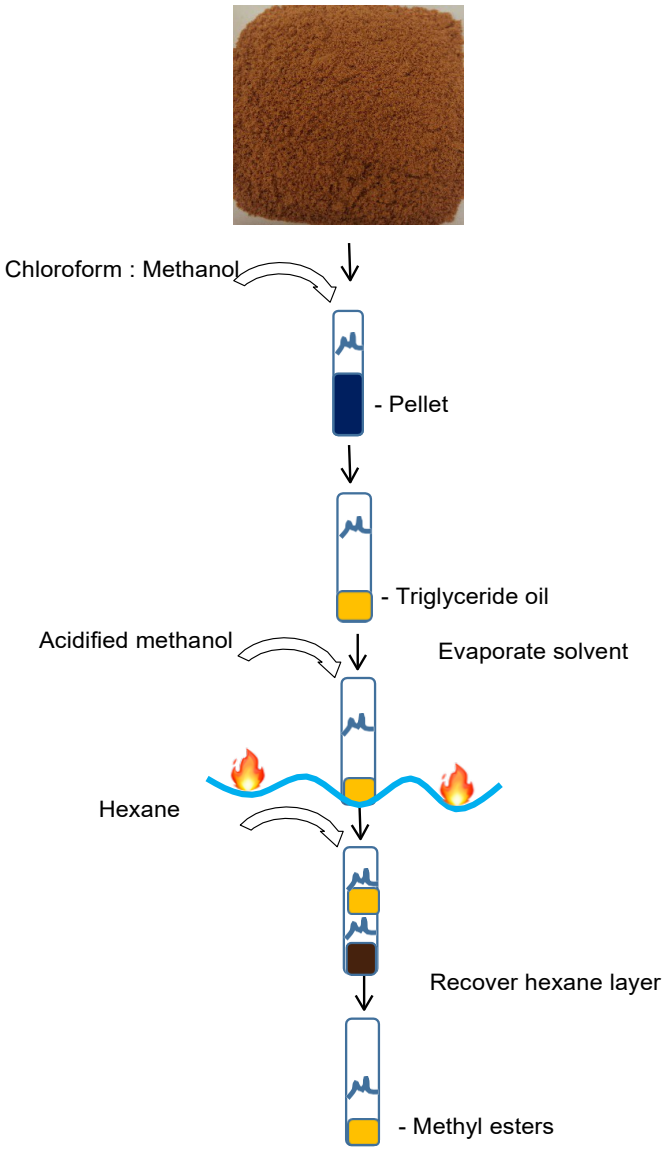



**Figure 3. Schematic representation of elution and methyl ester production methods. Dried, powdered complex was weighed into glass 8 ml tubes, mixed with solvent , then centrifuged. The eluted pellet was discarded and the recovered solvent was evaporated to yield recovered triglyceride oil. The recovered triglyceride oil was mixed with acidified methanol and heated at 90°C for one hour to produce methyl esters. The cooled acidified methanol was mixed with equal volume of hexane to recover methyl esters. The recovered methyl esters were then quantified.**

**Analysis of Long-Chain Fatty Acid Methyl Esters**. The n-3 PUFA methyl esters produced from samples and standards were analyzed using an Agilent Technologies GC-MS (6890N GC,5973N MS, 7683 injector) equipped with a 60-m × 0.25 mm DB- XLB fused silica capillary column (J&W Scientific, Folsom, CA, USA). Ultra-high purity helium (UHP300, Airgas,

Radnor, PA, USA) was used as the carrier gas with a constant flow of 1.3 ml/min. Samples (2.0 µl) were injected in split mode with a ratio of 20:1 with an injection flow rate of 25 ml/min. Chromatographic parameters consisted of injection port at 280 ˚C, oven at 50 ˚C for 5 min followed by a ramp at 15 ˚C/min to 280 ˚C followed by a hold time of 10 min at 280°C with MS scanning from 50 to 800 amu. The retention time of EPA was 24.39 min and of DHA was 26.57 min. The Wiley 7N Library was used for verifying peak identity in addition to the retention time of the standards. The concentrations of the fatty acids were calculated from the integrated peak areas of the methylated fatty acids relative to standard curves created from methylated chemical standards of DHA and EPA (Sigma Chemicals, St. Louis, MO). Automated background subtract was used to eliminate background signal noise.

**Project 2: Recovery of n-3 PUFAs from storage under reductive conditions**

**Materials**. Materials for this experiment were identical to the ones used for project1, however due to consistency issues with the MEG-1812, it was decided to change the TG oil source. A menhaden fish oil purchased from VWR International (Radnor, PA, USA) acted as the TG oil source for project 2.

TG oil-fortified WPI and control sample were prepared in the same manner as they were in project 1. However, to minimize exposure to atmospheric oxygen during the drying process, all samples were condensed under rotoevaporation and dried to a powder under lyophilization.

**Experimental Design**. All samples were aliquoted in equal amounts and stored in light protected, sealed glass scintillation vials under nitrogen conditions in anaerobic chambers and placed at 4°C, room temperature (approx. 20°C), and 37°C. Nitrogen conditions were achieved by adding dry ice to the chamber before sealing, removing oxygen from

the chamber through vacuum, then adding nitrogen gas to the chamber. Pressure inside the container was maintained at 10-15 PSI according to the pressure valve attached to the chamber. TG oil stability measurements and production of methyl esters were done in a manner consistent with the methods described in project 1. Three separate scintillation vials were sampled once at each collection point creating triplicate measurements per dried complex or oil at each collection point. Additionally, no vial was sampled more than once. Elution of triglyceride oil, derivatization of methyl esters, and methyl ester recovery quantification was performed identically to procedures in experiment 1.

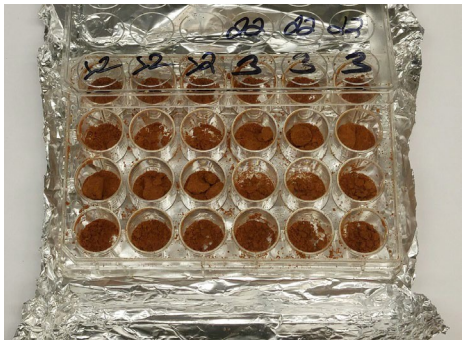
a.  b. 

**Figure 4. Differences in storage set-up between oxidative and reductive projects. Under oxidative conditions (a.), dried, powdered complex was aliquoted in equal amounts into a 24 multiwell plate pictured above. The plate was labeled, taped shut, and wrapped in foil, then kept in a dark space at 4°C, 20°C or 37 °C. Under reductive conditions (b.), the dried, powdered complex was aliquoted in equal amounts into foil wrapped glass scintillation vials, placed into the anaerobic chamber pictured above, and kept under nitrogen conditions (described in Experimental Design above) at 4°C, 20°C or 37 °C.**

**Project 3: Recovery of n-3 PUFAs from potential commercial application**.

**Materials.** DHA standard cis-4,7,10,13,16,19-Docosahexanoic acid (≥98%), EPA standard cis-5,8,11,14,17-Eicosapentaenoic acid (≥99%), hydrochloric acid (HPLC grade, 1N) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hexane (HPLC grade 95% n-hexanes) was purchased from Thermo-Fisher Scientific (Hampton, NH, USA). Methanol (HPLC grade) was purchased from VWR International (Randor, PA, USA). Whole blueberries and ground fish meal were mixed to produce four different wet

weight ratios of fish meal to blueberries (outlined in table 1), then co-dried at 40°C. Co-dried blueberry - fish meal samples were prepared and provided by Talsa (Lima, Peru).

| Sample | Fish meal: Blueberry (%) |
|---|---|
| M1 | 80:20 |
| M2 | 20:80 |
| M3 | 40:60 |
| M4 | 60:40 |

**Table 2. Percentage of fish meal: blueberry by wet weight of each sample.**

**Experimental Design**. Equal amounts of each co-dried blueberry – fish meal sample was placed into cell culture multiwell plates. Each plate was covered and taped closed, wrapped in aluminum foil to protect from light, and placed at 4°C, room temperature (approx. 20°C), and 37°C. For stability measurements, a 300 mg aliquot from three separate wells per individual co-dried blueberry: fish meal sample was taken at each collection point producing a set of triplicate measurements per sample set per collection point. Each well was sampled once at each collection point and no well was sampled more than once

To elute the n-3 PUFAs, produce and quantify methyl esters from the fish meal the same methods applied in project 1 were applied here.

**Statistical Analysis.**

Graphic representation of recoverable EPA and DHA are shown in Figures 5 - 8. Each graph demonstrates the mean percent of the individual n-3 PUFAs that were elutable from the protein-polyphenol at each time point. Triplicate samples were processed and measured using gas chromatography-mass spectrometry. Calculations were done based upon area under the curve measurements of recoverable methyl esters at baseline. Graphs represent mean percent recovery of triplicate samples compared to baseline.

Standard error and percent recovery was calculated based upon triplicate sample percent recovery compared to baseline (time point zero) percent recovery. GraphPad Prism 8.0 was used to perform ordinary two-way ANOVA analysis and Tukey's multiple comparison test, with a single pooled variance on mean percent recovery at each time point between treatments. Statistical significance between treatments at each time point is determined by a p-value $<0.05$.

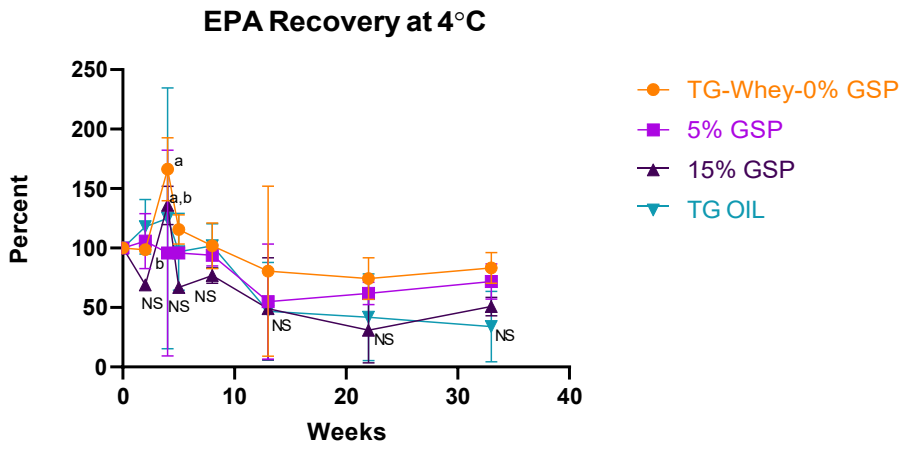
## Results

### Recovery of n-3 PUFAs from storage under oxidative and reductive conditions.

Samples stored under conditions similar to expected post-consumer purchase storage conditions demonstrated a decrease in recoverable EPA and DHA methyl esters compared to samples stored under conditions similar to on-shelf, pre-consumer purchase. No significant differences in methyl ester recovery for either EPA or DHA were observed between all treatments at the study term, 33 weeks, when samples were stored under oxidative conditions (fig. 5 and 6). Loss of detectable EPA and DHA methyl esters was observed at 20°C and 37°C and loss was accelerated as storage conditions became more aggressive (fig. 5b., 5c., 6b., and 6c.).

Significant differences in recoverable EPA and DHA methyl esters between protein-polyphenol treatments and free TG oil were observed from samples stored at 4°C under reductive conditions (fig. 7a and 8a.). No statistical significance in recoverable EPA and DHA methyl esters was observed between treatments at study term, 30 weeks, when stored at 37°C (fig. 7c and 8c). When stored at 20°C no statistically significant differences in recoverable EPA and DHA methyl esters were observed between 5% GSP

samples and free TG oil at study term however, 0% GSP recoverable methyl esters were significantly higher than free TG oil at 30 weeks (fig. 7b and 8b). Consistent among all the data, is a seeming increase in methyl ester recovery above 100%. This may be due to a biochemical change in the protein-polyphenol complex that allows for better elution of the TG oil and may be attenuated under reductive conditions

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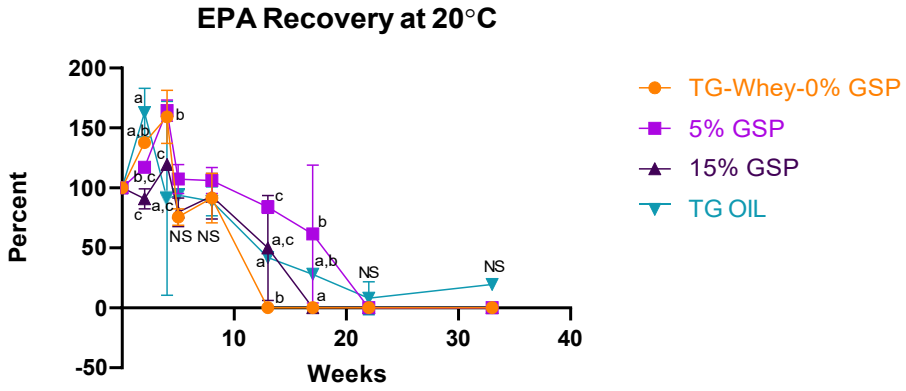


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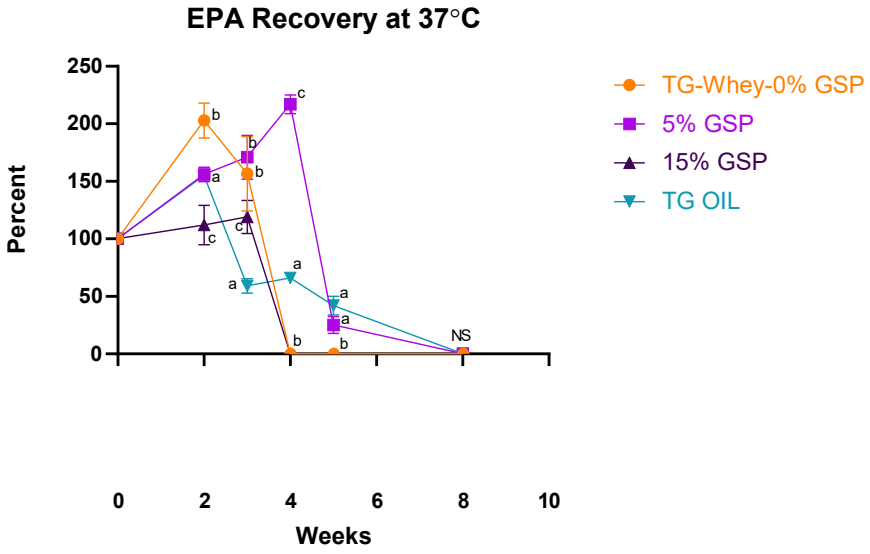
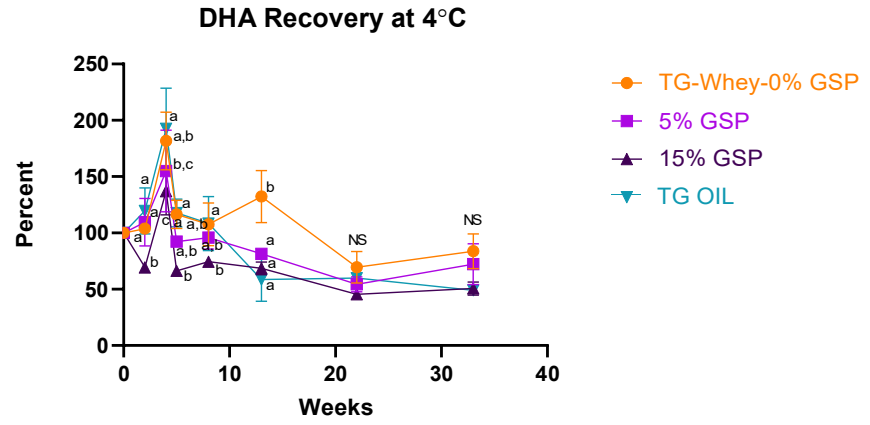



c.

**Figure 5. Graphical representation of mean percent recovery of EPA stored under oxidative conditions. (a). Percent recovery of EPA at 4°C. (b). Percent recovery of EPA at 20°C. (c). Percent recovery of EPA at 37°C. Ordinary two-way ANOVA analysis and Tukey's multicomparison test used to determine differences in methyl ester recovery between treatments at each time point. NS indicates no significance between treatments at the given time point.**

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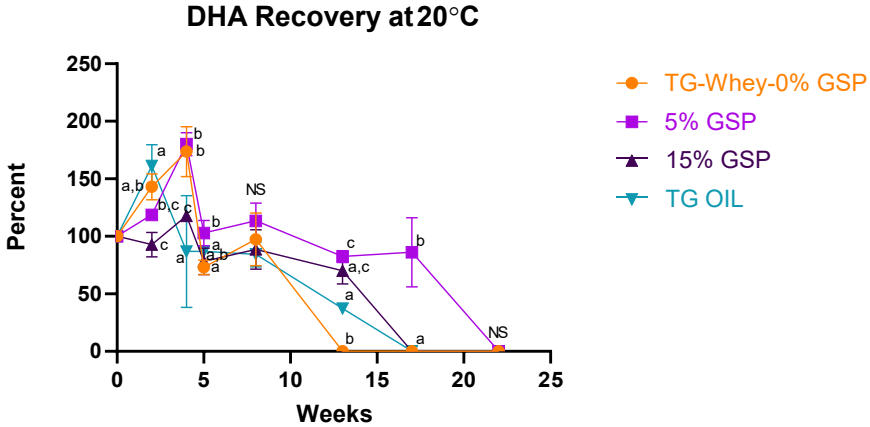


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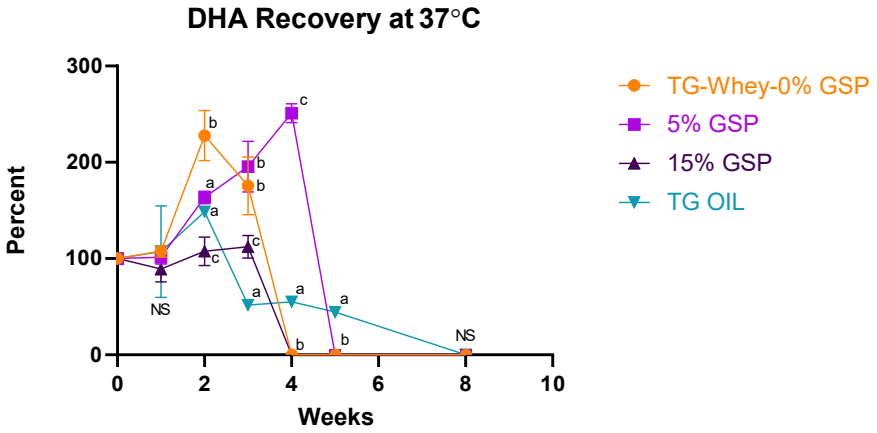
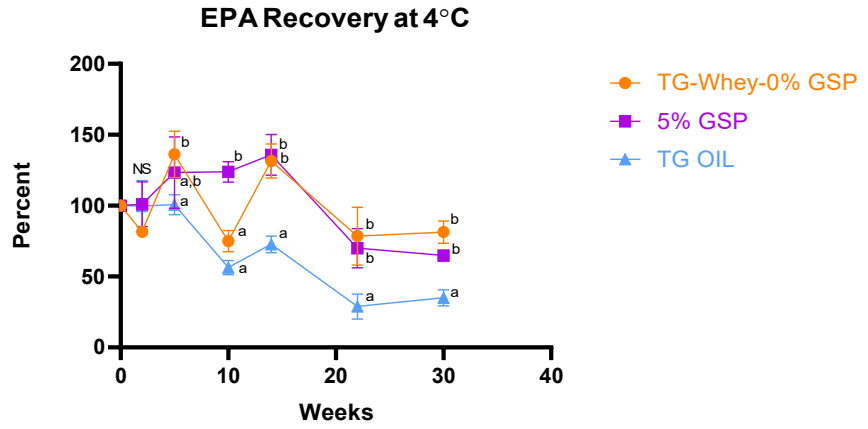



c.

**Figure 6. Graphical representation of mean percent recovery of DHA stored under oxidative conditions. (a). Percent recovery of DHA at 4°C. (b). Percent recovery of DHA at 20°C. (c). Percent recovery of DHA at 37°C. Two-way ANOVA analysis and Tukey's multicomparison test used to determine differences in methyl ester recovery between treatments at each time point. NS indicates no significance between treatments at the given time point.**

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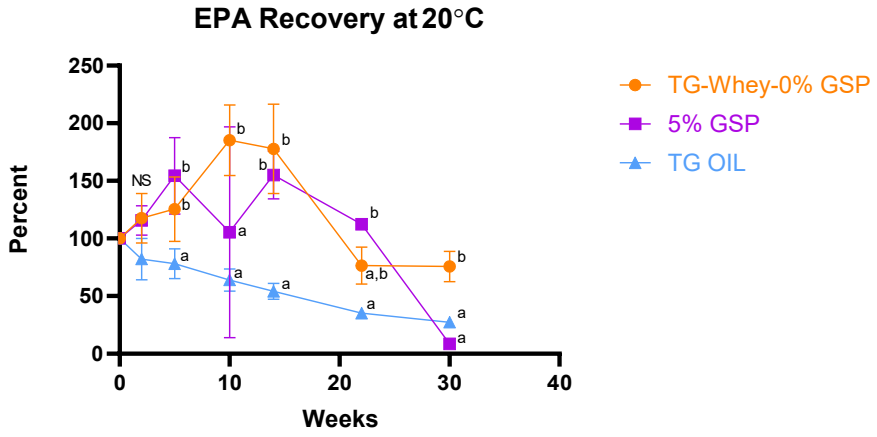


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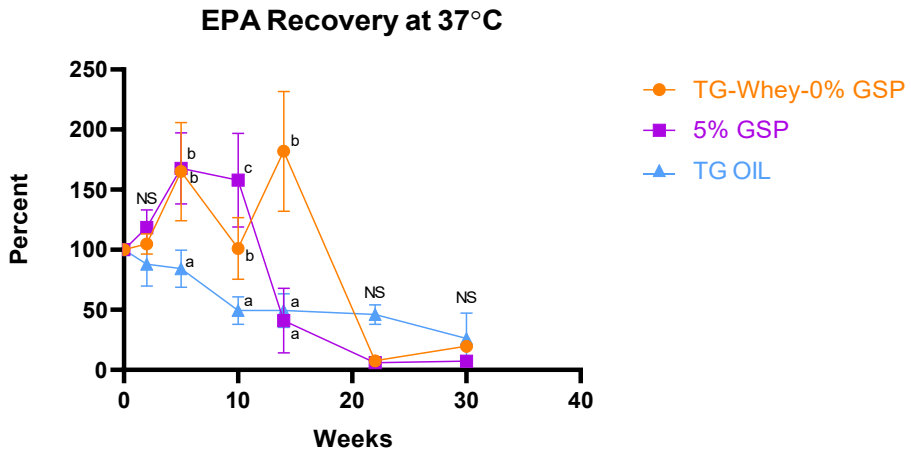
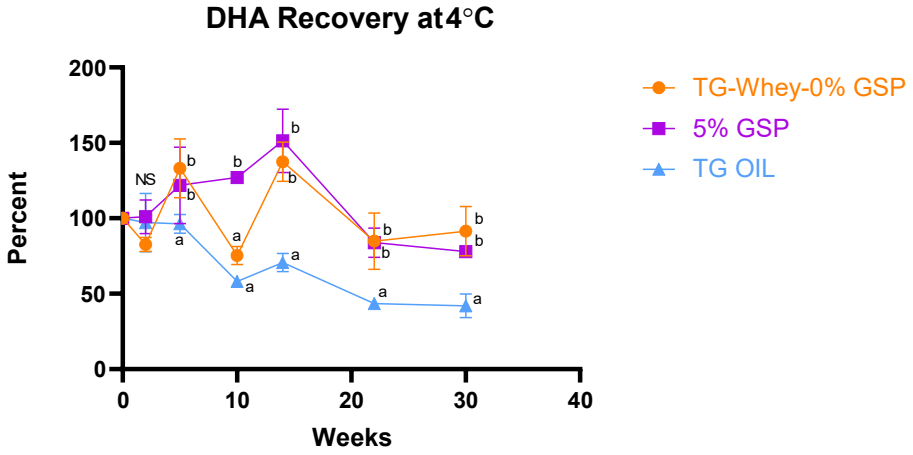
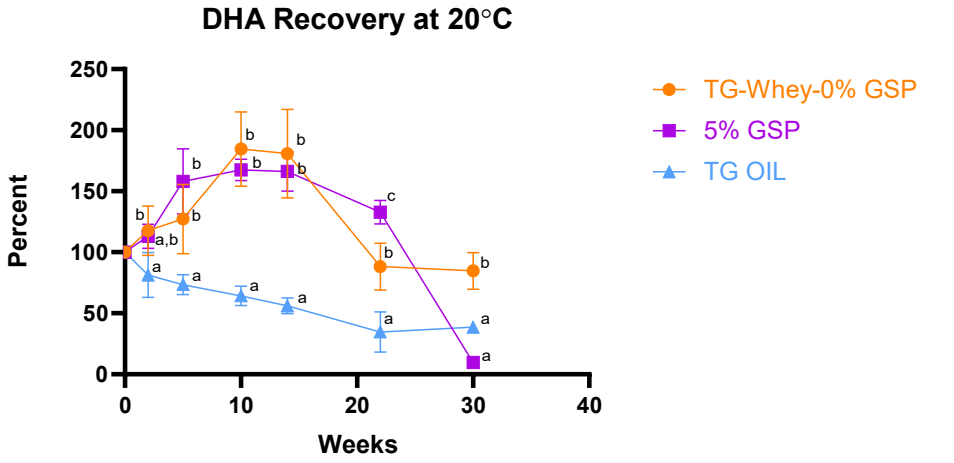



c.

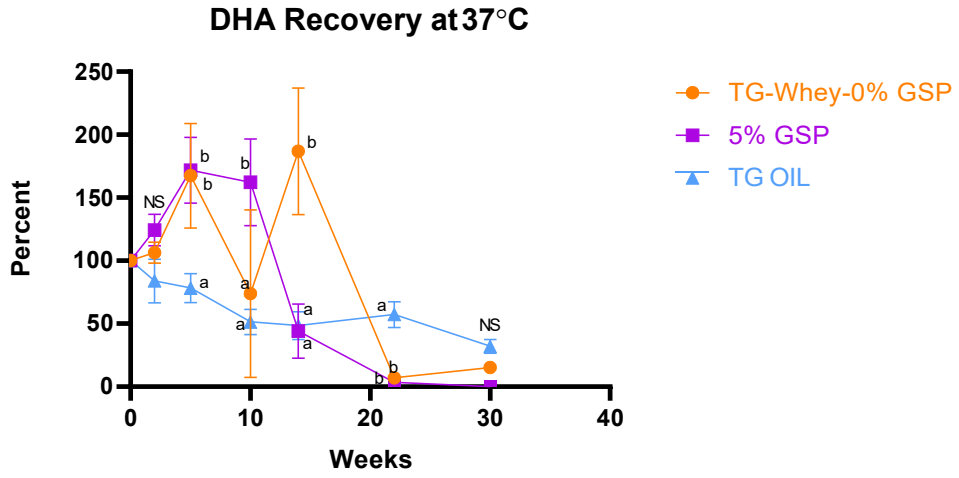
**Figure 7. Graphical representation of mean percent recovery of EPA stored under reductive conditions. (a). Percent recovery of EPA at 4°C. (b). Percent recovery of EPA at 20°C. (c). Percent recovery of EPA at 37°C. Two-way ANOVA analysis and Tukey's multicomparison test used to determine differences in methyl ester recovery between treatments at each time point. NS indicates no significance between treatments at the given time point.**




a.




b.




c.

**Figure 8. Graphical representation of mean percent recovery of DHA stored under reductive conditions. (a). Percent recovery of DHA at 4°C. (b). Percent recovery of DHA at 20°C. (c). Percent recovery of DHA at 37°C. Two-way ANOVA analysis and Tukey's multicomparison test used to determine differences in methyl ester recovery between treatments at each time point. NS indicates no significance between treatments at the given time point.**

## Recovery of omega-3 fatty acids from blueberry: fish meal commercial samples.

The wet weight composition (by percentage) of the blueberry: fish meal of each sample can be seen in table 1. No statistically significant differences in recoverable EPA methyl esters were observed between samples at 4°C and 37°C or for DHA methyl esters at 20°C (fig 9a., 9c., and 9e.) at 18 weeks. Statistically significant differences in DHA methyl ester recovery was observed between M1 and M2 at 4°C, M4 and M2 at 37°C (fig. 9d., and 9f.) and for EPA methyl ester recovery between M2 and M4 at 20°C (fig.

9b.) at 18 weeks. However, when considering concentrations of recoverable methyl esters, no significant differences between samples were observed for EPA under all three storage conditions at 18 weeks (fig. 10a., 10b., and 10c.), but significant differences were observed at study term for recoverable DHA methyl esters at all three storage conditions (fig. 10d., 10e., 10f.).

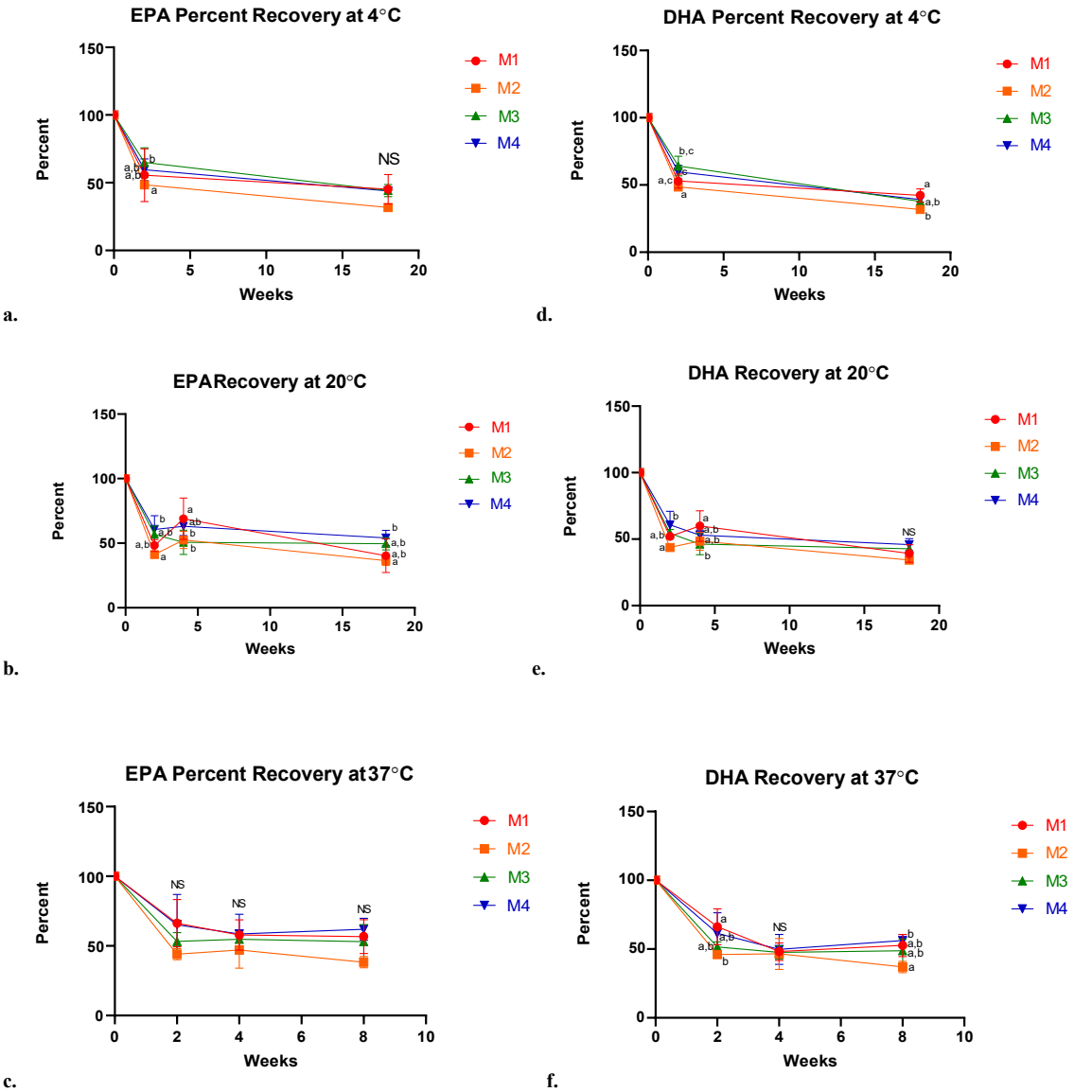



**Figure 9. Graphical representation of n-3 PUFA from storage under oxidative conditions. (a). Percent recovery of EPA at 4°C. (b). Percent recovery of EPA at 20°C. (c). Percent recovery of EPA at 37°C. (d). Percent recovery of DHA at 4°C. (e). Percent recovery of DHA at 20°C. (f). Percent recovery of DHA at 37°C. Two-way ANOVA analysis and Tukey's multicomparison test used to determine differences in methyl ester recovery between treatments at each time point. NS indicates no significance between treatments at the given time point**

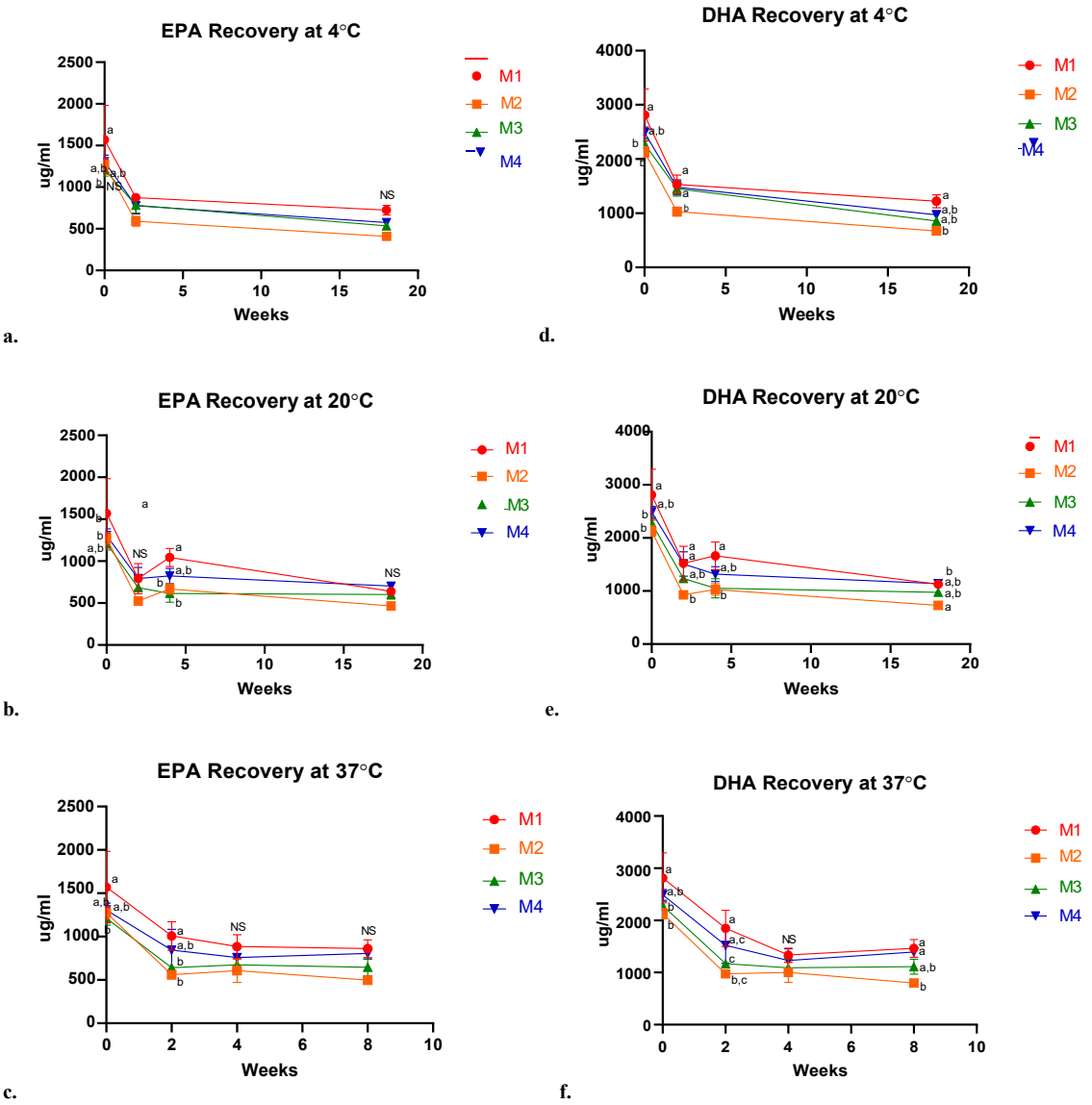



**Figure 10. Graphical representation of n-3 PUFA whole value recovery from storage under oxidative conditions. (a). Percent recovery of EPA at 4°C. (b). Percent recovery of EPA at 20°C. (c). Percent recovery of EPA at 37°C. (d). Percent recovery of DHA at 4°C. (e). Percent recovery of DHA at 20°C. (f). Percent recovery of DHA at 37°C. Two-way ANOVA analysis and Tukey's multicomparison test used to determine differences in methyl ester recovery between treatments at each time point. NS indicates no significance between treatments at the given time point.**

## Discussion

Some positive associations between a diet high in n-3 PUFAs and health exist in the published data. However, the average western diet is lacking in n-3 PUFA rich foods, specifically oily fish, and conversion of plant derived ALA to EPA and DHA is

inefficient. Therefore, n-3 PUFAs supplementation is recommended. Unfortunately, most n-3 PUFAs supplements are derived from marine sources and delivered in the form of encapsulated triglyceride oil. These oils are prone to rapid oxidation, limiting shelf-life storage. Additionally, the strong "fishy" after taste of the supplements is an aspect many consumers find unsavory. The purpose of this project was to determine a mechanism that can decrease the rate of marine derived n-3 PUFAs oxidation, ultimately increasing shelf-life stability while delivering a high dosage of supplemental PUFAs in a dry form.

There is an increased consumer demand to use natural antioxidants as opposed to synthesized antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) in food and supplement production[14.] Grape seeds are a waste product of juice and wine production. These seeds are high in polyphenol content, 5-8% depending upon variety[15].

Production of the TG oil enhanced complexes was done based upon weight of final product. As total polyphenol content increases, the amount of protein added to the GSE – TG oil mixture will decrease while total weight of TG oil added remains the same. Therefore, a 5% total polyphenol-protein complex has more protein by weight compared to a 15% total polyphenol-protein complex. In these experiments the protein content, as a percentage of final weight was: 75%, 59.9%, 29.7% for 0%, 5%, and 15% GSP (for oxidative conditions samples) respectively and 75%, and 61.1% for 0% and 5% GSP (for reductive conditions samples) respectively. This difference in protein could have had an effect on surface area available for TG oil binding or coating. The 15% GSP complex

was determined to be ineffective and too expensive to produce commercially, so it was omitted from the reductive conditions experiment.

Under oxidative conditions no significant differences in recoverable EPA and DHA methyl esters were observed between all treatments at study term regardless of environmental conditions. The progressive loss of detectable EPA and DHA methyl esters observed at 20°C and 37°C across all treatments indicates an effect of environmental conditions on n-3 PUFA recovery that is independent of treatment.

When placed under reductive storage conditions the 5% GSP complex and 0% GSP complex performed superiorly to the free TG oil at 4°C at study term, 30 weeks. However, when stored at 20°C differences between recoverable methyl esters from 5% GSP complex and free TG oil were not statistically significant at study term while differences between recoverable methyl esters from the 0% GSP complex was significantly different than both 5% GSP and free TG oil. Interestingly, percent recovery of EPA and DHA methyl esters was highest from the 0% GSP complex. This indicates a potential stability effect offered by protein rather than polyphenols. Protein isolates have been demonstrated to protect against lipid peroxidation by forming microencapsulation with the fatty acids, enhancing oxidative stability and attenuating the characteristic odor[40].

Due to technical issues with the MEG-3 1812 oil, the choice to change TG oil sources was made, therefore, the TG oil used in these projects were different. The MEG-1812 used in the oxidative conditions project was provided by DSM and was produced from mostly sardines (as was stated on the label and paperwork). However, the total EPA and DHA content of the TG oil claimed on the certificate of analysis and analytical protocol

provided by DSM did not match the total EPA and DHA content detected in the early rounds of experimentation. Additionally, TG oil stocks were exhausted upon completion of sample production for oxidative conditions. Therefore, it was decided to source a TG oil from a commercial supplier. The menhaden oil was used for the reductive conditions experimental analysis. This difference in oil could account for the differences observed in n-3 PUFA recovery. However it would be likely that, despite differences in TG oil source or potential differences in quality, the trend in n-3 PUFA methyl ester recovery would be unchanged.

The blueberry: fish meal samples were provided by Tulsa, a commercial agribusiness based in Lima, Peru. The aim of this project was to determine if whole blueberries could prevent the oxidation of n-3 PUFAs in fish meal if the blueberries and fish meal were co-dried. The samples were prepared in Peru by Talsa. Whole blueberries (that were deemed inadequate for commercial sale) were mixed with fish meal in various ratios based upon wet weight, and co-dried at 40°C, packaged as a dried sample, and shipped to Rutgers. The samples arrived in separate Ziploc bags in a regular, sealed manila envelope after an approximately 6 week delay in both Peruvian and United States customs and were ground to a powder in the lab. Each sample arrived labeled with the corresponding percentage of blueberry: fish meal however neither a blueberry nor fish meal sample control was provided. Therefore, it is hard to conclude based upon comparison between n-3 PUFAs methyl ester recovery of the samples that the statistical significance observed at some storage conditions is nothing more than a reflection of the amount of fish meal present in the sample as opposed to a protective affect offered by the antioxidants in the

blueberries. Regardless, it was demonstrated that the ratio of fish meal to blueberry content did not have a strong effect on recoverable n-3 PUFAs methyl esters.

**Conclusion**

These data suggest that while there may not be a protective effect of polyphenols exclusively on recoverable n-3 PUFA methyl esters, that an interaction between protein isolate and n-3 PUFAs could reduce oxidation of the marine derived fatty acids at room temperature and 4°C under reductive conditions. It is possible that the combination of polyphenols, protein, and n-3 PUFAs in a dried formula could enable the delivery of marine derived n-3 PUFAs that is shelf stable. However extracted polyphenols without a minimum amount of protein are not sufficient to offer protection against n-3 PUFA oxidation in a dried complex.

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