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Utilizing the parts of common food products as functional ingredients Part 1: Separation and concentration of ω -3 PUFA-rich phospholipids by hydration of krill oil Part 2: Functional properties of a concentrated egg yolk protein powder using a one-step organic solvent extraction process

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Utilizing the parts of common food products as functional ingredients

Part 1: Separation and concentration of ω -3 PUFA-rich phospholipids by hydration of krill oil

Part 2: Functional properties of a concentrated egg yolk protein powder using a one-step organic solvent extraction process

Casey Showman

A thesis submitted to the
Davis College of Agriculture, Forestry, and Design
at West Virginia University
In Partial fulfillment of the Requirements
for the degree of
Master of Science
in
Nutritional and Food Sciences

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ABSTRACT

Utilizing the parts of common food products as functional ingredients: Part 1: Separation and concentration of ω -3 PUFA-rich phospholipids by hydration of krill oil Part 2: Functional properties of a concentrated egg yolk protein powder using a one-step organic solvent extraction process

Casey Showman

The food industry separates food products into different parts to better utilize the functional and nutritional benefits of each fraction. The overall focus of this thesis is on separating and utilizing fractions and co-products from food manufacturing as functional ingredients. Therefore, the objective of this research was to 1) evaluate the effectiveness of water degumming to separate omega-3 PUFA-rich phospholipids from other lipids found in krill oil and 2) determine how different organic solvents would affect physiochemical and functional properties of egg yolk protein recovered from spray-dried egg yolk using a novel one-step organic solvent extraction method.

The aim of the first study was to use the principles of water degumming to separate phospholipids from triglycerides in krill oil by hydration to concentrate them in a gum fraction. Water was mixed with krill oil (KO) in ratios of 100% KO, 75:25 KO:H₂O, 50:50 KO:H₂O, and 25:75 KO:H₂O wt/wt. Separation occurred only in 75:25 KO:H₂O and 50:50 KO:H₂O ratios. 75:25 KO:H₂O showed the optimal separation of lipid classes with a more concentrated phospholipid content ($67.6 \pm 1.97\%$) when compared to the 50:50 KO:H₂O ($49.73 \pm 3.90\%$; $p < 0.05$) and contained negligible amounts of the neutral lipids. The fatty acid profile for 75:25 KO:H₂O gum and oil fraction showed a significant difference ($p < 0.05$) in EPA and DHA content. Gum fractions containing phospholipids were further analyzed by HPLC-Mass spectrometry. HPLC-MS results showed a presence of PC(20:5/20:5), PC(20:5/22:6), and PC(22:6/22:6) in the gum fractions of both 75:25 KO:H₂O and 50:50 KO:H₂O ratios. This

simple method demonstrates that the principles of water degumming can be applied to separate neutral and polar lipids present in KO.

A second study sought to determine how different organic solvents would affect properties of egg yolk protein concentrate recovered from spray-dried egg yolk using a novel one-step organic solvent extraction method. Solvents tested were hexane-isopropanol (HI) (3:2, v:v), chloroform-methanol (CM) (2:1, v:v), and methyl-tert-butyl ether (MTBE). Egg yolk protein concentrates contained a greater concentration of protein compared to initial egg yolk powder ($p < 0.05$). Solubility of the initial egg yolk powder and protein concentrates was consistent among all samples obtained when HI and MTBE were used as the extraction solvent ($p > 0.05$). A significant decrease in solubility occurred from 20% to 4% protein ($p < 0.05$) when CM was used. It is likely the polarity of the methanol that denatured proteins and altered functionality such as the ability to gel. Gels from MTBE and HI protein concentrates had a similar hardness ($p > 0.05$), but were harder than initial egg yolk powder gels ($p < 0.05$). MTBE gels were springier, more cohesive, and gummier ($p < 0.05$) with similar resistance to initial egg yolk powder gels ($p > 0.05$). The gels made from protein concentrates were less yellow ($p < 0.05$) than initial gels due to fat soluble pigments removed with lipid extraction. This method shows MTBE has the potential to lower lipid content while increasing protein content of egg yolk powder as a functional food product for human consumption.

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CHAPTER I

INTRODUCTION

Many food products on grocery store shelves contain food additives that are added to enhance flavor, shelf stability, nutrition profile, and customer acceptability. The rampant development of food additives was sparked by the changes in large scale food production (WHO, 2018). These ingredients often started out as co-products or byproducts from manufacturing other food products. Some examples include; lecithin from soybean oil refinement and whey protein from the dairy industry. Industries developed ways to utilize these products to reduce waste while improving the quality of food products. Other products, such as lecithin from egg yolk, are separated into fractions to take advantage of their functional properties and further utilize their nutritional profile.

Lecithin is the commercial name for phospholipids; mainly phosphatidylcholine. Their functionality is due to the amphiphilic nature of the polar head group and two fatty acid tails. The majority of lecithin on the market is sourced from refining soybean oil. Soybean lecithin is comprised of over 50% omega-6 fatty acids (Stankova et al., 2013). This class of fatty acids is associated with risks of cardiovascular and inflammatory diseases (Simopoulos, 2002). The risks can be lowered by incorporating higher amounts of long chain omega-3 fatty acids into the diet. Krill oil (*Euphausia superba*) is often marketed as an omega-3 supplement. It contains most of the omega-3 fatty acids bound to phospholipids with the phospholipid content greater than 50% of total lipids (Castro-Gomez et al., 2015; Kolakowska, 1991; Winther et al., 2010). Therefore, the first part of this thesis focuses on using the same principles employed in soybean lecithin

production to isolate the fraction of krill oil concentrated with phospholipids and omega-3 fatty acids for the potential use as a food additive.

Egg yolk is also used as a food additive and is an excellent source of nutrients while also providing functional properties to foods such as mayonnaise, cakes and pastries, and many other products. Lecithin is also extracted from egg yolk, but the methodology differs from that of obtaining soybean lecithin. Organic solvent lipid extraction is often used to separate lecithin from egg yolk, and the remaining co-product contains a significant amount of protein. This protein is altered or damaged due to the nature of the solvents changing the structure of the lipoproteins in order to remove lipids (Kovalcuks and Duma, 2014). Since the protein structure is altered, the functionality that egg yolk is known for is greatly decreased (Marcet et al., 2017). Therefore, the second part of this thesis focuses on determining how various organic solvents would affect proximate composition, water solubility, and gelling of egg yolk protein concentrate recovered from spray-dried egg yolk after a novel one-step organic solvent extraction method.

References

1. Castro-Gomez, M., Holgado, F., Rodriguez-Alacala, L., Montero, O., Fontecha, J. (2015). Comprehensive study of the lipid classes of krill oil by fractionation and identification of triglycerides, diacylglycerols, and phospholipid molecular species by using UPLC/QToF-MS. 8:2568-2580.
2. Kolakowska, A. (1991). The influence of sex and maturity stage of krill (*Euphausia superba* Dana) upon the content and composition of its lipids. Polish Polar Research. 12:73-78.
3. Kovalcuks, A. and Duma, M. (2014). Solvent extraction of egg oil from liquid egg yolk. 9th Baltic Conference on Food Science and Technology- Food for Consumer Well-Being: Foodbalt 2014:253-256.
4. Marcet, I., Saez, S., Rendueles, M., Diaz, M. (2017). Edible films from residual delipidated egg yolk proteins. Journal of Food Science and Technology. 54(12):3969-3978.
5. Simopoulos, A. P. (2002). The importance of the ratio of omega-6/omega-3 essential fatty acids. Biomedicine & pharmacotherapy. 56(8):365-379.
6. Stankova, B., Kremmyda, L. S., Tvrzicka, E., & ŽÁK, A. (2013). Fatty Acid Composition of Commercially Available Nutrition Supplements. Czech Journal of Food Science, 31(3).
7. Winther B, Hoem N, Berge K, Reubsaet L (2010) Elucidation of phosphatidylcholine composition in krill oil extracted from *Euphausia superba*. Lipids 46:25-36.
8. World Health Organization (WHO). Food Additives. 2018. <http://www.who.int/news-room/fact-sheets/detail/food-additives>

PART ONE: Separation and concentration of ω -3 PUFA-rich phospholipids by hydration of krill oil

CHAPTER II

REVIEW OF LITERATURE

Introduction

The lipid and fatty acid composition of krill oil (*Euphausia superba*) is well known, and the benefits of the oil as a nutritional supplement have been extensively studied. Krill oil has an abundance of omega-3 fatty acids and a majority of these are found bound to phospholipids. The phospholipid content is greater than 50% of total lipids and dependent on many factors that influence lipid composition of the species (Castro-Gomez et al., 2015; Kolakowska, 1991; Winther et al., 2010). Phospholipids have many physiological and functional properties that are due to the amphiphilic nature of the polar head and fatty acid tails. The amphiphilic nature of the molecule allows phospholipids to function as an emulsifier, wetting agent, solubilizer and form micelles and liposomes. The commercial product, lecithin, is often a byproduct of soybean oil production or extracted from egg yolk. Soybean and other vegetable lecithin is a byproduct obtained through the process of water degumming; one of the first steps in oil refining. The process calls for mixing heated water with crude oil to precipitate phospholipids and other unwanted parts. Because of the amphiphilic nature, the polar heads can absorb water and become oil insoluble. The phospholipids are easily separated by centrifugation, dried, and prepared for product use. Water degumming is an effective method for refining oils as well as producing a functional food additive. The aim of this review was to characterize krill oil lipid and fatty acid profiles and to investigate water degumming principles as a method to separate phospholipids.

Omega-3 polyunsaturated fatty acids

Omega-3 polyunsaturated fatty acids (ω -3 PUFAs) are fatty acids in which the first double bond appears at the third carbon from the methyl end of the fatty acid carbon chain. These fatty acids are vital for visual and cognitive development as well as the prevention of cardiovascular disease (Uauy et al., 2003). Long chain unsaturated fatty acids also play a role in fluidity of cellular membranes. The ω -3 fatty acid, alpha-linolenic acid (ALA), cannot be synthesized by the human body, and therefore is considered an essential fatty acid. From the dietary intake of ALA, long chain (LC) PUFAs such as eicosapentaenoic acid (EPA, 20:5 ω -3) and docosahexaenoic acid (DHA, 22:6 ω -3) can be synthesized by the body through a series of elongations and desaturations; however, this conversion is limited because of competition with linoleic acid (LA) conversion and ALA catabolism for energy rather than EPA and DHA conversion (Arterburn et al., 2006). Dietary recommendations for the intake of ω -3 LCPUFAs which can be achieved by consuming fatty fish twice per week (Harris et al., 2008). Cold water fish are rich in EPA and DHA due to their diet of marine algae and crustaceans such as krill.

Dietary consumption of ω -3 PUFAs is an influencer on the cellular membrane fatty acid composition, and therefore influences membrane functionality (Hulbert et al., 2005). The second carbon of the glycerol backbone, the sn2 position, of a phospholipid typically contains an ω -3 or ω -6 fatty acid which are cleaved by pancreatic phospholipase A₂ and play a role in inflammatory responses (Gijon et al, 1994). The type of fatty acid cleaved also impacts prostaglandin formation. Prostaglandins synthesized from unsaturated fatty acids act as hormones and exert stimulatory effects throughout the body. Omega-3 fatty acids, commonly EPA, are substrates for 3-series prostaglandins while ω -6 fatty acids, like arachidonic acid (AA), are substrates for 2-series prostaglandins. Incorporation of DHA in the brain and other tissues of piglets was studied by Liu and colleagues (2014) which found that DHA bound to phospholipids had higher efficacy

of delivery than triglycerides. Pigs have a similar metabolism to humans and are used as a model in nutrition studies (Liu et al., 2014).

Omega-3 PUFAs EPA and DHA are found mainly bound to phospholipids in krill oil while fish oil contains EPA and DHA exclusively bound to triglycerides. There are many studies that looked at the effects of krill oil supplementation on a number of health issues, fatty acid incorporation in cellular membranes, and krill oil versus fish oil supplementation. Results indicate krill oil supplementation reduces inflammation responses by decreasing the conversion of AA to series-2 prostaglandins (Deutsch, 2007). Krill oil will reduce total cholesterol and LDL while increasing HDL in patients with hyperlipidemia when paired with drug therapy (Gurevich et al., 1997) or taken alone as a supplement (Bunea et al., 2004). Bunea et al. (2004) also reported that at equal doses, krill oil was more effective than fish oil at serum lipid levels reduction in a human randomized clinical trial. In another study, lipid serum levels were tested after krill and fish oil supplementation, and it was reported that both oils resulted in similar reductions of triglyceride and total cholesterol levels in rats when compared to a control group (Ferramosca et al., 2012). Krill oil is a beneficial source of omega-3 fatty acids, especially with a majority bound to phospholipids.

Phospholipids

Phospholipids are a class of lipids with amphiphilic properties due to their structure. They play an important role in biological membranes and many body functions. The molecule consists of a hydrophilic polar phosphate head and two lipophilic fatty acid tails. The tails consist of a wide range of fatty acids, but typically contain a saturated and unsaturated fatty acid attached to sn1 and sn2, respectively (Berdanier, 2008). Phospholipids are synthesized in the human body or consumed through diet. Over 90% of phosphatidylcholine (PC), the predominant class of

phospholipids, efficiently digested and absorbed by the intestines. They are not only found as functional molecules in cellular membranes and physiological processes, but are also functional additives in food and pharmaceutical products.

There are several classes of phospholipids present in oils which includes phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidic acid (PA). The classes are separated based on the chemical structure of the group attached to the phosphate head. Hydratability of the phospholipid is dependent on the hydrophilicity of the type of phosphatide (Dijkstra, 2018). PC is fully hydratable due to the chemical structure, swelling capacity, and ordered structure formation (Diez, 1995 and Larsson, 1994). The head group on a PC molecule carries a large dipole moment due to the negative charge on the phosphate and positive charge on the trimethylamine group which allow PC to strongly favor water (Dijkstra, 2017). On the other hand, the charges on a PE head group have a small dipole moment and therefore, isn't easily hydrated by water at neutral pH (Dijkstra, 2017). The class of phospholipid also determines the behavior of the molecule when mixed in oil and water. PC takes on a more lamellar layer while PE will form a reverse micelle structure (van Nieuwenhuyzen, 2015).

Characterization of krill oil lipid and fatty acid profiles

Antarctic krill (*Euphausia superba*) are a species of crustacean that contain significant amounts of ω -3 PUFAs, specifically EPA and DHA (Kolakowska et al., 1994). Lipid and fatty acid content of krill oil depends on many factors such as harvest season, diet, age, extraction procedures, and analysis methods (Saether et al., 1986; Kolakowska, 1991). Krill oil is often encapsulated and marketed as an omega-3 supplement. Supplementation of PUFA-rich products has gained interest for use as an additive to increase the nutritive profile of foods. Work done by

Le Grandois and colleagues (2009) determined the PC molecular species profiles of common PC rich food matrices including soy, egg yolk, ox liver, and krill oil. Krill oil contained the most diverse fatty acid profile and the highest amount of ω -3 PUFAs, specifically 34% EPA and 16% DHA. The most abundant PC molecular species in krill oil was PC16:0-20:5 (32%). This study found there were PC molecules containing EPA and DHA located at the sn1 and sn2 of the same molecule. PC20:5-20:5 made up 1.7%, PC20:5-22:6 was 2.3%, and PC22:6-22:6 was 0.8% of the identified molecules. Authors claim this study as the first to identify PC species found in krill, and develop a successful liquid chromatography- mass spectrometry method to separate and identify molecules in food products rich in PC (Le Grandois et al., 2009).

Winther and others (2011) employed a combination of HPLC and MS techniques in order to further elucidate PC species found in Antarctic krill oil and verified findings from the previously mentioned study by Le Grandois et al. (2009). The PC content of the krill oil was 34% sample with a total of 51 choline containing phospholipids with fatty acids on the sn1 and sn2 positions. Of those, 58% contained an ω -3 fatty acid on one position and 10% on both positions of the molecule. PC20:5-20:5, PC20:5-22:6, and PC22:6-22:6 were all identified in the krill oil used in the experiment with PC20:5-20:5 having the highest relative intensity of the three. Winther and colleagues (2011) detected a greater number of PC species and fatty acids with longer acyl chains than the previous study conducted by Le Grandois et al. (2009), however, the krill oil was from different suppliers and could have had different compositions.

Since the ω -3 PUFAs are held by phospholipids in krill oil, the previous studies focused on the characterization of the polar lipids (Le Grandois et al., 2009 and Winther et al., 2011). Castro-Gomez and other (2015) aimed to characterize fatty acid profiles and molecular species found in mono-, di- and triglycerides (TAG) as well as phospholipids (PL). The authors utilized

ultra-performance liquid chromatography-mass spectrometry to identify composition of lipids. Samples were comprised of 44% TAG and 49% PL with minor amounts of other lipids. Three fractions were obtained based on polarity. Fraction 1 contained all TAG. Fraction 2 contained diglycerides (DAG), cholesterol, and free fatty acids. The third fraction contained phospholipids (99% PC). The PC rich fraction had a significantly higher ω -3 PUFA content when compared to the other two fractions ($p < 0.05$) (Castro-Gomez, 2015). This indicated that EPA and DHA are mainly associated with PL. The PL fraction also displayed lower SFA/PUFA and ω -6/ ω -3 ratios when compared to the TAG-rich fraction (0.85 vs 4.65 and 0.08 vs 0.38, respectively) (Castro-Gomez, 2015). The authors identified 28 different PC species and also confirmed the presence of PC20:5-20:5, PC20:5-22:6, and PC22:6-22:6 with a combined content up to 6.5% of PC species. Results of this study provide insight into the complex composition of krill oil and confirm the high degree of unsaturation of fatty acids associated with phospholipids (Castro-Gomez, 2015).

The bioavailability of ω -3 PUFAs differs depending on the associated lipid class due to digestion processes in the body (Schuchardt and Hahn, 2013). Due to the amphiphilic nature of phospholipids, they are not dependent on bile salts to form micelles for lipid digestion. Intestinal absorption of phospholipids is much higher than triglycerides as well. Up to 20% of dietary phospholipids can be absorbed passively in the intestine (Zierenberg and Grundy, 1982). There are a variety of ω -3 PUFAs supplements on the market with different lipid compositions, therefore Kutzner and colleagues (2016) analyzed fatty acid profiles and their distribution in lipid classes of 12 products including krill oil, fish oil, algal oil, and plant oil. Fatty acid distribution among krill oil lipid classes indicated 67-71% of ω -3 PUFAs were bound to PL while they were mainly associated with triglycerides in fish oils and other samples. In krill oil, over 54% of the ω -6 fatty acids were bound to triglycerides and less than 39% were associated

with PL. The results of the study show differences in fatty acid profiles as well as lipid class distribution in a variety of ω -3 PUFAs supplements which plays a role in bioavailability of the product (Kutzner et al., 2016).

These studies show the complexity of the lipid and fatty acid profiles of krill oil. Unlike other types of oil, phospholipids in krill oil are not seen as an impurity because of the distribution of highly unsaturated fatty acids. Comparison of the results from each study are difficult because of different krill oil sources and methodologies for characterization, but the researchers built off each other to provide more information about the components of krill oil. Information shows the phospholipid fraction is a significant portion of krill oil and if concentrated could be utilized as functional food or pharmaceutical product.

Water degumming principles and optimization

Water degumming is one of the steps in oil refining for seed and vegetable oils in order to remove phospholipids and other unwanted compounds. The process consists of mixing a predetermined amount of water with oil to transform the phospholipids into an oil-insoluble gum for removal. About 80-95% of phospholipids are removed from oil by water degumming (List et al., 1978). The amount of water added in the practice is critical, and is dependent upon the amount of phospholipids present. The amount of water added in degumming ranges between 75% of the phospholipid content up to equivalent amounts of phospholipid, by weight, equaling out to 1-5% water, by weight of oil, added in the degumming processes of canola, soybean, and sunflower oils. (Braae, 1976 and Brekke, 1987). Too little or too much water will cause phospholipids to remain in the oil phase or make separation of the phases difficult (Brekke, 1987). The temperature of water and oil and speed of agitation employed in the process also plays a role in efficacy of degumming.

List et al. (1981) tested a wide range of degumming conditions in order to optimize the removal of lecithin from soybean oil with 1.74% phospholipids. The researchers tested four different parameters including time, water concentration, agitation, and temperature. Each parameter consisted of four variations based on previously reviewed literature. Four degumming replicates were completed for each parameter in order to test all variations while keeping others constant. List and others (1981) found that the hydration of phospholipids is rapid and over 90% of them are removed from oil within 5 minutes and there was little increase in phospholipid removal when mixed for 60 minutes. Temperature and agitation caused little variation in the amount of phospholipids removed from the soybean oil, although temperature effected the color of the lecithin. Higher temperatures (75 and 90°C) produced a darker lecithin which is unwanted by manufacturers. They determined water concentration had the greatest effect on the amount of gum removed from oil. Water concentrations lower and higher than the phospholipid content removed less than 90% of phospholipids and caused the gums to contain high amounts of oil. Adding 2% water, roughly equal to amount of phospholipids in the oil, resulted in the most efficient degumming parameter (List et al., 1981). The authors suggested that the optimal conditions for the water degumming of soybean oil include 15 min of agitation at a 400 rpm, an oil temperature of 60 °C, and a water amount close to the amount of phospholipids in the crude oil (List et a., 1981).

The kinetics of separating phospholipid classes by degumming was studied by Pan and others (2000). Crude sunflower seed oil was subjected to water degumming (2.5% water) at 40 °C for 5 to 55 minutes. Acid degumming was also performed on the water degummed oil with 2.5% phosphoric and citric acid solution. Water degumming removed over 98% of the phosphatidylcholine species after 5 minutes, and the optimal removal of hydratable

phospholipids (PC, PI, and PE) occurred after 35 minutes of mixing. Acid degumming removed the nonhydratable phospholipids in the water degummed oil. At 35 minutes, the water-degummed oil had the lowest residual phosphorous content which is related to more of the phospholipid classes removed at that time period. Hydratable phospholipids are related to the chemical structure, water absorption, and structure formation of the phospholipid species. PC is an easily hydratable phospholipid as indicated by the results. This study provides evidence of the kinetics of removing different classes of phospholipids by water and acid degumming (Pan et al., 2000).

Work completed by Lei and other (2003) developed and analyzed a ternary phase diagram of soybean oil-soybean phosphatidylcholine (PC)-water to understand the water degumming process. The authors used a combination of theoretical models and experimental observations of oil particles to describe the behaviors of oil and phospholipids in the presence of different amounts of water. From observations and theoretical explanations, Lei et al. (2003) found that the water to PC ratio should be 2:3 or about 34-40% based on water and PC. At this ratio, water is absorbed and large flocculates are formed, which are oil insoluble and denser so they are easily separated by centrifugation. The amount of water added to the system influences phospholipid particle formation. When the optimal amount of water is added to the system the phospholipids take on a normal structure, the head groups absorb more water, and aggregate to form large flocculates (Lei et al., 2003). At lower water concentrations, phospholipids form liposomes in an inverted structure (head group in, fatty acid tails out) and are dispersed in oil (Lei et al., 2003). When there is excess water in the system, it penetrates the liposomes and flocculates creating smaller particles that are dispersed in water again (Lei et al., 2003). The

results from the study by Lei et al (2003) indicate there is an optimal amount of water to mix with oil to easily separate the phospholipids.

Patent US 8828447 B2 filed by Soerensen and Jensen (2014) for Polar Omega outlined a process to isolate phospholipids from a fish oil to obtain a fraction containing polyunsaturated fatty acids (PUFAs). It was invented because of the need for a large scale production of a phospholipid product (Soerensen and Jensen, 2014). An overview of the invention calls for mixing a polar solvent with a phospholipid-rich fish oil, centrifugation, and separation of the polar fraction from the lipid fraction. The inventors used fish oil as a generic term to encompass any marine species oil that would contain both neutral and polar lipids. The polar solvent used to fractionate the phospholipids should be oil insoluble and create the two phases mentioned earlier with the optimal solvent being water (Soerensen and Jensen, 2014). The inventors suggested a ratio of solvent to oil to be between 5:95 and 25:75 to avoid excess solvent and entrapment of neutral lipids (Soerensen and Jensen, 2014). The ratio should be based on the polar lipids present in the fish oil. The temperature of mixing was suggested to be above ambient temperatures to optimize extraction, but this may damage the lipids. The patent provided examples that showed the polar fraction had an increased phospholipid content compared to the fish oil (>40% vs ~16%) as well as an increased PUFA content (Soerensen and Jensen, 2014). Patent US 8828447 B2 filed by Soerensen and Jensen (2014) developed a method to fraction fish oil into a polar lipid fraction with the intent of using water to isolate PUFAs.

It is concluded that the amount of water mixed with oil in the process plays a significant role in the efficacy of the removal or separation of phospholipids. Suggestions for the amount of water to use in the methodology range from a water to PC ratio of 2:3 up to equal amounts of water to phospholipid content. These numbers are dependent on other factors such as other types

of phospholipids, temperature of the oil, and speed of agitation. Most of the research examines the principles of water degumming and process optimization of plant oils and sources of lecithin such as soybeans. These types of crude oils contain only small amounts of phospholipids when compared to the content of krill oil.

Krill oil contains a significant amount of ω -3 PUFAs bound to phosphatidylcholine (PC) species. Based on the review of hydrating phospholipids, PC is fully hydratable by water degumming. The success of water degumming is highly dependent of the amount of water added to oil in the process. Since krill oil contains high amounts of PC, the principles of water degumming could be utilized to separate and concentrate ω -3 PUFAs bound to phospholipid into a fraction that could be used as nutritional and functional product.

References

1. Arterburn, L.M.m., Hall, E. B., Oken, H. (2006). Distribution, interconversion, and dose response of n-3 fatty acids in humans, *American Journal of Clinical Nutrition*. 83:1467S-1476S.
2. Berdanier, C. D. Fatty acids and membrane function. Edited by Chow, C. K. *Fatty Acids in Foods and their Health Implications* (3rd edition). pp. 693-755. Boca Raton, Fl. CRC Press Taylor & Francis Group.
3. Braae, B. (1976). Degumming and refining practices in Europe. *Journal of American Oil Chemists' Society*. 43:353-357.
4. Brekke, O. L. (1987). Oil degumming and soybean lecithin, chapter 5. Edited by Erickson, D. R., Pryde, E. H., Brekke, O. L., Mounts, T. L., Falb, R. A. *Handbook of Soy Oil Processing and Utilization*. American Soybean Association and American Oil Chemists' Society, Champaign.
5. Bunea, R., El Farrah, K., Deutsch, L. (2004). Evaluation of the effects of Neptune krill oil on the clinical course of hyperlipidemia. *Alternative Medicine Review*. 9(4):420-428.
6. Castro-Gomez, M., Holgado, F., Rodriguez-Alacala, L., Montero, O., Fontecha, J. (2015). Comprehensive study of the lipid classes of krill oil by fractionation and identification of triglycerides, diacylglycerols, and phospholipid molecular species by using UPLC/QToF-MS. 8:2568-2580.
7. Deutsch, L. (2007). Evaluation of the effect of Neptune Krill Oil on chronic inflammation and arthritic symptoms. *Journal of the American College of Nutrition*. 26(1):39-48.
8. Diez, R. (1995) Superdegumming of crude oils. *Aceitas Grasas*. 20:294-307.
9. Dijkstra, A. J. (2017). Hypothesis paper: about water degumming and the hydration of non-hydratable phosphatides. *European Journal of Lipid Science and Technology*. 119:1600496.
10. Dijkstra, A. J. (2018). Edible oil processing: introduction to degumming. *AOCS Lipid Library*. Retrieved April 4, 2018, from <http://lipidlibrary.aocs.org/OilsFats/content.cfm?ItemNumber=40325>
11. Ferramosca, A., Conte, A., Burri, L., Berge, K., De Nuccio, F., Giudetti, A.M., Zara, V. (2012). A krill oil supplemented diet suppresses hepatic steatosis in high-fat fed rats. *PLoS One*. 7:e38797.
12. Gijon, M. A., Leslie, C. C. (1994). Regulation of arachidonic acid release and cytosolic phospholipase A₂ activation. *Journal of Leukocyte Biology*. 50:330-336.
13. Gurevich, V., Bondarenko, B. B., Gundermann, K. J., Schumacher, R., Astashkina, T., Ivanov, V., Popov, Y., Shatilina, L., Kazennova, N. (1997). Polyunsaturated phospholipids increase the hypolipidemic effect of Lovastatin. *European Journal of Internal Medicine*. 13–18.
14. Harris, W. S., Kris-Etherton, P. M., Harris, K. A. (2008) Intakes of long-chain omega-3 fatty acid associated with reduced risk for death from coronary heart disease in healthy adults. *Current Atherosclerosis Reports*. 10(6):503-509.

15. Hulbert, A. J., Turner, N., Storlien, L. H., Else, P. L. (2005) Dietary fats and membrane function: implications for metabolism and disease. *Biological Reviews of the Cambridge Philosophical Society*. 80(1):155-169.
16. Kolakowska, A. (1991). The influence of sex and maturity stage of krill (*Euphausia superba* Dana) upon the content and composition of its lipids. *Polish Polar Research*. 12:73-78.
17. Kolakowska, A., Kolakowski, E., & Szczygielski, M. (1994). Winter season krill (*Euphausia superba* D.) as a source of n-3 polyunsaturated fatty acids. *Molecular Nutrition & Food Research*, 38(2): 128-134.
18. Kutzner, L., Ostermann, A.I., Konrad, T., Riegel, D., Hellhake, S., Schuchardt, J.P., Schebb, N.H. (2016). Lipid class specific quantitative analysis of n-3 polyunsaturated fatty acids in food supplements. *Journal of Agriculture and Food Chemistry*. 65:139-147.
19. Larsson, K. (1994). Liquid crystalline lipid-water phases. *Lipids, Molecular Organization, Physical Functions and Technical Applications*. 62.
20. Le Grandois, J., Marchioni, E., Zhao, M., Giuffrida, F., Ennahar, S., & Bindler, F. (2009). Investigation of natural phosphatidylcholine sources: separation and identification by liquid chromatography– electrospray ionization– tandem mass spectrometry (LC– ESI– MS2) of molecular species. *Journal of agricultural and food chemistry*, 57(14):6014-6020.
21. Lei, L., Ma, Y., Kodali, D. R., Liang, J., Davis, H. T. (2003). Ternary phase diagram of soybean phosphatidylcholine-water-soybean oil and its application to the water degumming process. *Journal of American Oil Chemists' Society*. 80:383-388.
22. List, G. R., Avellaneda, J. M., Mounts, T. L. (1981). Effect of degumming conditions on removal and quality of soybean lecithin. *Journal of American Oil Chemists' Society*. 58:892-898.
23. Liu, L., Bartke, N., Van Daele, H., Lawrence, P., Qin, X., Park, H. G., Kothapalli, K., Windust, A., Bindels, J., Wang, Z., Brenna, J. T. (2014). Higher efficacy of dietary DHA provided as a phospholipid than as a triglyceride for brain DHA accretion in neonatal piglets. *Journal of Lipid Research*, 55(3), 531–539.
24. Pan, L. G., Campana, A., Toms, M. C., et al. (2000). A kinetic study of phospholipid extraction by degumming process in sunflower seed oil. *Journal of American Oil Chemists' Society*. 77(12):1273-1276.
25. Schuchardt, J. P., and Hahn, A. (2013). Bioavailability of long-chain omega-3 fatty acids. *Prostaglandins, Leukotrienes and Essential Fatty Acids*. 89(1):1-8.
26. Saether, O., Ellingsen, T.E., Mohr, V. (1986) Lipids of North Atlantic krill. *Journal of Lipid Research*. 27:274-285.
27. Soerensen H. O., Jensen, N. C. (2014). United States Patent US8,828,447 B2. Washington, DC: U.S. Patent and Trademark Office.
28. Uauy, R., Hoffman, D. R., Mena, P., Llanos, A., Birch, E. E. (2003). Term infant studies of DHA and ARA supplementation on neurodevelopment: results of randomized controlled trials. *The Journal of Pediatrics*, 143(4):17-25.

29. Van Nieuwenhuyzen, W. (2015). Production and utilization of natural phospholipids. Edited by Ahmad, M. U. and Xu, X. Polar lipids: biology, chemistry, and technology. Elsevier. 245-276.
30. Winther B, Hoem N, Berge K, Reutsaet L (2010) Elucidation of phosphatidylcholine composition in krill oil extracted from *Euphausia superba*. *Lipids* 46:25-36.
31. Zierenberg O, Grundy SM. (1982). Intestinal absorption of polyenephosphatidylcholine in man. *J Lipid Res.* 23(8):1136–1142.

CHAPTER III

Separation and concentration of ω -3 PUFA-rich phospholipids by hydration of krill oil

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Abstract

Water degumming is an industrial process used to separate phospholipids in crude oil. Antarctic krill oil (KO) (*Euphausia superba*) has a significant amount of phospholipids with bound ω -3 PUFAs, specifically eicosapentaenoic acid (EPA, 20:5 ω -3) and docosahexaenoic acid (DHA, 22:6 ω -3). The aim of this study was to use the principles of water degumming to separate phospholipids from triglycerides in krill oil by hydration to concentrate them in a water fraction. Water was mixed with krill oil (KO) in ratios of 100% KO, 75:25 KO:H₂O, 50:50 KO:H₂O, and 25:75 KO:H₂O wt/wt. Samples were centrifuged to separate the water soluble and insoluble fractions, and freeze-dried. Fractions were analyzed for specific lipid classes by thin layer chromatography (TLC) with densitometry. Fatty acid profiles were determined by gas chromatography-flame ionization detector (GC-FID). Separation into gum and oil fractions occurred only in the 75:25 KO:H₂O and 50:50 KO:H₂O samples. For both ratios, TLC and densitometry showed that the gum fractions contained all phospholipids within those samples. The optimal separation of lipid classes occurred in the 75:25 KO:H₂O with a more concentrated phospholipid concentration of $67.6 \pm 1.97\%$ compared to $49.73 \pm 3.90\%$ ($p < 0.05$) in the 50:50 KO:H₂O sample. The 50:50 KO:H₂O gum fraction contained $23.88 \pm 6.36\%$ triglycerides while the 75:25 KO:H₂O contained negligible amounts of the neutral lipids. The oil fractions for both 75:25 KO:H₂O and 50:50 KO:H₂O contained mainly triglycerides ($>80\%$). The fatty acid profile of the 75:25 KO:H₂O fractions were significantly different ($p < 0.05$) in EPA and DHA content. The gum fraction contained $30.97 \pm 2.34\%$ EPA and $19.82 \pm 1.69\%$ DHA while the oil fraction contained $14.12 \pm 1.16\%$ and $6.47 \pm 0.90\%$, respectively. Gum fractions containing phospholipids were further analyzed by HPLC-Mass spectrometry. HPLC-MS results showed the presence of PC(20:5/20:5), PC(20:5/22:6), and PC(22:6/22:6) in the gum fractions of both 75:25 KO:H₂O

and 50:50 KO:H₂O ratios. This simple method demonstrates that the principles of water degumming can be applied to separate neutral and polar lipids present in KO. The concentrated phospholipids, which contain high amounts of EPA and DHA, have potential to be further utilized as lecithin in food products or in the pharmaceutical industry.

KEYWORDS: omega-3 fatty acids, phospholipids, water degumming

Introduction

Cardiovascular disease is the leading cause of death in the United States (Bunea et al., 2004). Omega-3 polyunsaturated fatty acids (ω -3 PUFAs) have potential health benefits such as protection against cardiovascular disease (Bunea et al., 2004). Specific ω -3 PUFAs of importance include eicosapentaenoic acid (EPA, 20:5 ω -3) and docosahexaenoic acid (DHA, 22:6 ω -3). Typical sources of EPA and DHA are fatty fish; however, krill also contains significant amounts of these PUFAs.

Antarctic Krill (*Euphausia superba*) are small crustaceans with one of the largest biomasses of any multicellular animal species (Nicol et al., 1987). The lipid content, on a dry matter basis, ranges from 12-50% based on the species, maturity, and season of harvest (Saether et al., 1986; Kolakowska, 1991). Krill are an unusual species due to the composition of lipids in their bodies. The phospholipid composition of krill oil varies among publications ranging from 33%-49% (Castro-Gomez et al., 2015; Gigliotti et al., 2011; Winther et al., 2010; Xie et al., 2017) to 46-64% of the total lipid composition (Kolakowska, 1991). Compared to fish oils, the ω -3 PUFAs in krill oil are mainly associated with phospholipids rather than triglycerides (Winther et al., 2010). For example, Kutzner et al. (2016) reported that 67-71% of ω -3 PUFAs are bound to phospholipids. Interestingly, these specific ω -3 PUFAs will appear incorporated together on a single phospholipid. Castro-Gomez et al. (2015) reported EPA/DHA and EPA/EPA was present on the sn1 and sn2 positions of the phospholipid. The molecules had a relative content of 6.5% of the total phosphatidylcholine content.

Phospholipids are most often found within cell membranes. Their structure consists of a polar, hydrophilic phosphate head and two fatty acid tails that are hydrophobic. The polarity and hydrophobic interactions allow for the formation of lipid bilayers (Gilbert, 2000). The level of

saturation of the fatty acid tails can vary. Unsaturated fatty acids allow the bilayer to be more fluid in colder temperatures because cis-double bonds create kinks which inhibit the tails from lining up closely (Mathews et al., 2000). Saturated fatty acid tails would cause the lipid bilayer to be too rigid in the colder temperatures of the krill's environment.

Phospholipids have several functions in the pharmaceutical and food production industries due to their amphiphilic character. Phospholipids, specifically phosphatidylcholine, are commonly called lecithin. One of the most common uses of lecithin in the food industry is for stabilizing oil/water emulsions (Ceci et al., 2008). In the pharmaceutical industry, phospholipids serve as drug delivery systems because of their unique biocompatibility and hydrophilic properties (Li et al., 2014). Studies show that dietary phospholipids have a high bioavailability and can have a protective effect on diseases such as coronary heart disease (Küllenberg et al., 2012).

There are several classes of phospholipids present in oils which includes phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidic acid (PA). The classes are separated based on the chemical structure of the group attached to the phosphate head and makes them either hydratable or nonhydratable. Nonhydratable phospholipids are commonly separated from oil and neutral lipids through processes such as acid degumming, whereas hydratable phospholipids are separated with the addition of water (Dijkstra and Segers, 2007). PC, the main phospholipid in krill oil, is fully hydratable due to its chemical structure, swelling capacity, and ordered structure formation (Diez, 1995 and Larsson, 1994). The head group on a PC molecule carries a large dipole moment due to the negative charge on the phosphate and positive charge on the trimethylamine group which allow PC to strongly favor water (Dijkstra, 2018). Water

degumming is an industrial process to separate phospholipids in crude oil to make commercial lecithin. In the degumming process, the amount of water added is typically equal to that of the amount of gum to be separated (Braae, 1976). The process includes heating the oil and adding water which hydrates the polar lipids causing the oil and gum to separate resulting in a refined oil that consists of triglycerides and gum that contains the phospholipids. This process has not been tested for the separation of phospholipids from krill; therefore, the aim of this study was to use the principles of water degumming to separate phospholipids from triglycerides in krill oil by hydration. It is hypothesized that a majority of ω -3 PUFAs will be found in the water fraction associated with phospholipids.

Materials and Methods

Sample

Virgin krill oil from Antarctic krill (*Euphausia superba*) was purchased from Jedwards International, Inc. (Braintree, MA, U.S.A.). Upon arrival, the krill oil (KO) was stored in an amber glass bottle, flushed with nitrogen, and stored at 2-5 °C throughout the experiment.

Hydration fractionation

Virgin krill oil was fractionated using deionized water (ddH₂O). Various ratios of krill oil and ddH₂O were used. Hydration trials included 100% KO, 75:25 KO:H₂O, 50:50 KO:H₂O, and 25:75 KO:H₂O wt/wt. Krill oil and ddH₂O were weighed to a final volume of 50 grams into zeroed glass beakers, flooded with nitrogen, and covered with foil. Each ratio was stirred at 200 RPM for 24 hours at room temperature (~20 °C). After 24 hours, each sample was placed into a centrifuge tube. Water soluble and insoluble fractions were separated by centrifuging at 5000 g for 5 min at 22 °C. Following centrifugation, visual fractions were manually decanted into freeze

dry cups. Fractions were then freeze dried (VirTis, Model #35L) to remove H₂O and stored at -80 °C. Each ratio was completed in triplicates. Weights were recorded after stirring, separation of each fraction, and freeze-drying to calculate the separation yields of each fraction.

Thin Layer Chromatography

Thin Layer Chromatography was applied to separate lipid classes based on polarity. Separated fractions from previous methods diluted to 5 mg/mL in a 1:1 chloroform:methanol solvent. Twenty µL of the mixture was pipetted onto a silica TLC plate (Merck TLC Silicagel 60 plates with 60 Å pore size). For the mobile phase, the plate was developed in a hexane: diethyl ether: acetic acid (80:20:1.5) for approximately one hour until the solution reached one inch from the top of the plate. After drying for 5 min, plates were sprayed with 50% sulfuric acid in water solution, and placed in a 120°C drying oven for 45 min to develop spots. Standards for mono-, di-, and triglycerides, phospholipids, cholesterol (Supelco, Bellefonte, PA), and free fatty acids (oleic acid, Nu Chek Prep, Elysian, MN) were also plated and ran for identification of each lipid class. TLC was applied to all fractions from each ratio tested.

Images of the plates were captured with Gel Doc XR+ gel imaging system (Bio Rad # 170-8170) using transilluminating white light and CCD camera. Identified lipid class bands were analyzed using Gel Doc XR+ Image Lab software. Densitometry values are expressed as percent lipid class (mean±SD) of the total bands detected within the lane.

Fatty acid analysis

Extraction

Bligh and Dyer (1959) method using chloroform-methanol (C:M) mixture (2:1 v/v) was followed to extract total lipids from each fraction. A 0.05g sample was used from each fraction

for analysis. Trizma/EDTA buffer (50mM, pH=7.4) and a chloroform: methanol: glacial acetic acid ((C:M:A); 400:200:3mL) solvent were added. Nonadecanoic acid (19:0) was added (125 μ L) as an internal standard for quantification of fatty acids. Samples were incubated at room temperature for 10 min, then centrifuged at 900x g (4000rpm) for 10 min at 10°C. The separated lower layer was filtered through a pre-rinsed Whatman 1-PS filter (Whatman 2200090). The upper layer was run through extraction methods using a 4:1 chloroform: methanol solvent, centrifuged to separate, and again the lower layer was filtered through same filter. Filtrates were flushed with nitrogen gas at 60°C to dry.

Methylation

The extracted lipid was transmethyalted according to Fritsche and Johnson (1990). Methylation occurred by adding 4% H₂SO₄, in anhydrous methanol, to lipid and incubating in water bath at 90°C for 60 min. Deionized distilled water was added to stop the reaction after incubation period. Chloroform was added to extract fatty acid methyl esters (FAME), and mixture was centrifuged at 900x g for 10 min at 10°C. The separated lower layer was filtered through anhydrous Na₂SO₄. The collected layer was dried with nitrogen gas at 60°C, diluted with isooctane, and stored at -20°C until analyzed.

Fatty acid profile and quantification

Fatty acid methyl esters were analyzed using a Varian CP-3800 Gas Chromatograph (Varian Analytical Instruments; Walnut Creek, California, U.S.A) equipped with a flame ionization detector (FID; Varian Inc., Walnut Creek, California, U.S.A.). A silica capillary column (100 m length, 0.25 mm diameter) was used to separate the FAMEs. A method of 140°C held for 5 min followed by a temperature ramp of 4°C/min to 220°C held for 15 min was

adopted; totaling to 85 min for each FAME separation. Temperatures were held at 270°C and 300°C for the injector and detector respectively. Identification of FAMES in sample was based on retention times compared to FAME 37 standard. The Star GC workstation version 6 software (Varian Inc., Walnut Creek, California, U.S.A.) was used to determine the peak area and relative amounts of each fatty acid in the samples.

HPLC-MS

Sample preparation

Phosphatidylcholine quantification of the gum fractions was accomplished by reverse phase UHPLC-Mass spectrometry. The methodology is similar to that developed by Winther et al. (2011). Briefly, each sample and a stock solution of PC free matrix were dissolved in 60:40 dichloromethane:methanol mixture to a concentration of 1mg/mL. Phosphatidylcholine 22:6/22:6 standard (Avanti Polar Lipids, Alabaster, AL) was used as an external calibrator. The standard was mixed into PC free matrix solution in a set of dilutions with concentrations of 1µg/mL, 5 µg/mL, 10 µg/mL, 50 µg/mL, 100 µg/mL. A calibration curve of chromatographic peak area to standard concentration was established from set of dilutions to quantify krill oil samples.

HPLC Setup and Conditions

Chromatographic separation was performed in reverse phase on a Thermo Accela UHPLC with an Agilent Eclipse XDB-C18 column. Column dimensions were 2.1 x 100mm. There were two mobile phases used to elucidate the phospholipids. Phase A consisted of 90% water with 10% formic acid while phase B consisted of 60% methanol, 40% acetonitrile, and

0.1% formic acid. A linear gradient of the two phases, similar to that of Winther et al. (2011), was used for separation of PC species. The mobile phase flow rate was set to 0.26 mL/min.

Mass Spectrometry

Detection of phosphatidylcholine species was obtained using a Thermo Q-Exactive with method setup, data acquisition, and quantification done by software Xcalibur 3.0 (Thermo Scientific, USA). The mass spectrometer ran at a voltage of 4.00 kV, sheath gas flow rate of 30 (arbitrary units), auxiliary gas flow rate of 5, and sweep gas of 5. Capillary temperature was set to 200°C. Mass spectrometry resolution was set at 70,000 (m/z 200) for full mass scan, and 17,500 for tandem mass. Mass scan range was set to 100-1000 m/z in profile mode. Parallel reaction monitoring analysis was used. Both the precursor ions and the fragment ions were selected to monitor the transition using the ratio of respective peak areas to quantify each phospholipid. Masses for each PC species, determined by previous studies (Castro-Gomez et al., 2015), were used to identify the molecules by mass spectrometry.

The precursor m/z window was set as ± 1 . A full scan of precursor ions was carried out. Then the selected precursor ions were subjected to high-energy collision dissociation (HCD), followed by the selection of specific product ions in the Orbitrap Mass Analyzer (Thermo Scientific, USA).

Statistical analysis

Hydration fractionation trials were all performed in triplicates ($n=3$). Duplicate TLC with densitometry and GC-FID measurements were performed for each triplicate fractionation trial. HPLC-MS was conducted once. SAS JMP Pro version 13 (SAS Institute Inc., North Carolina, USA) was used for all analyses. One-way analysis of variance (ANOVA) with post-hoc analysis

using Tukey-Kramer's test ($p < 0.05$) was used to determine statistical differences between ratios and fractions.

Results and Discussion

Fractionation

Fractionation into water soluble and insoluble fractions, or “gum” and “oil” fractions, respectively, occurred only in the 75:25 KO:H₂O and 50:50 KO:H₂O ratios. The 25:75 KO:H₂O ratio formed an oil/water dispersion and since no fractionation occurred after centrifugation, further results are not reported. When 50:50 KO:H₂O ratio was used to separate lipids, the starting weight of krill oil was 25 g. After separation, the weight of the oil fraction was 2.50 ± 1.36 g and the weight of the gum fraction was 20.53 ± 1.16 g, for a separation rate of 89% in the gum fraction. On the other hand, when 75:25 KO:H₂O ratio was used the starting weight of the krill oil was 37.5 g. After separation, the weight of the oil fraction was 11.78 ± 1.42 g and the weight of the gum fraction was 19.83 ± 2.28 g, for a separation rate of 63% in the gum fraction. The recovery of total krill oil at the end of the hydration process was $>84\%$. Loss of sample was due to the transfer from container to container in each step of the process because of the viscosity of krill oil.

There were visual differences in both gum fractions obtained. The 50:50 KO:H₂O gum fraction had a similar appearance to the emulsion-like mixture created by the 25:75 KO:H₂O ratio, while the 75:25 KO:H₂O gum fraction had a dark red and sludge-like appearance. This difference was likely due to the entrapment of oil in structures formed in the hydration of the 50:50 KO:H₂O sample rather than the precipitation of polar lipids.

Water is a polar solvent that is immiscible in oil; therefore, when it interacts with phospholipids they too become insoluble in the oil when hydrated. When there is an optimal ratio

of krill oil to water, the phospholipids form aggregates that are oil insoluble and can be separated from the oil (Lei et al., 2003). Lei et al. (2003) published a ternary phase diagram of soybean oil-water-soybean PC to understand the appropriate amount of water to be added during degumming. The researchers found that when an excess of water is used, above the phospholipid saturation point, water droplets penetrate liposomes and disperse in the oil. This is likely why the 25:75 KO:H₂O ratio had no separation, as well as the 50:50 KO:H₂O ratio was approaching the saturation point of the phospholipids found in krill oil.

Lipid class profiles

Based on thin layer chromatography (TLC) and densitometry, water fractionation of phospholipids from neutral lipids, such as triglycerides, was possible. TLC displayed how each major lipid class in krill oil fractionated after the addition of water (Figure 1). Densitometry gave the relative distribution of lipid classes within each fraction obtained. Data are reported in Table 1 and are expressed as % lipid class $\pm$ standard deviation of in that particular fraction. Initial krill oil, used as the control in the procedure, contained 19.78 ± 3.43 % triglycerides and 46.28 ± 0.32 % phospholipids. The concentration of phospholipids is consistent with reported ranges between 46-64% (Kolakowski, 1991, Castro-Gomez et al., 2015, and Xie et al., 2017). On the other hand, Gigliotti et al. (2011) found a much lower phospholipid content based on TLC densitometry of the extracted krill oils. This was likely due to different extraction methods performed in the study as well as lipid class variations based on the season the krill were harvested (Saether et al., 1986).

Undetectable amounts of phospholipids remained in the 50:50 and 75:25 KO:H₂O oil fraction, likely because the phospholipids in krill oil are comprised of >90% phosphatidylcholine (PC) which is fully able to absorb water (Table 1; Akanbi and Barrow,

2017; Castro-Gomez et al., 2015; Dijkstra and Segers, 2007; Zhou et al., 2012). In addition, the 75:25 KO:H₂O ratio gave rise to better separation of lipid classes with more concentrated phospholipids (approximately 67%) and undetectable amounts of triglycerides in the gum fraction; whereas the 50:50 KO:H₂O gum fraction had a less concentrated phospholipid content (approximately 49%) due to the presence of triglycerides that make up about 23% of the gum fraction. The oil fractions of both 75:25 KO:H₂O and 50:50 KO:H₂O ratios contained mainly triglycerides (>80%).

TLC and densitometry results showed that over half of the cholesterol was held in the gum fraction of the two ratios where fractionation occurred. This is seen qualitatively on the TLC plate (Figure 1). Cholesterol is a relatively nonpolar molecule, but does contain a polar hydroxyl group which may have interacted with water molecules and caused the cholesterol to separate into the gum fraction (Mathews et al., 2000).

Fatty acid profile

Major fatty acids (FA) found in the 100% KO, 75:25 KO:H₂O, and 50:50 KO:H₂O ratios and their respective fractions (water/oil) are listed in Table 2. These FA include C14:0, C16:0, C16:1, C18:1, C18:3 (ALA), C20:2, C20:5 (EPA), and C22:6 (DHA). Results are presented as % fatty acid in total fatty acids identified and quantified. There were significant differences in fatty acid profiles for each oil and gum fraction ($p < 0.05$).

For both the 50:50 KO:H₂O and 75:25 KO:H₂O samples, the gum fractions contained a greater concentration of EPA and DHA than the oil fractions ($p < 0.05$). Within all fractions and initial krill oil, the 75:25 KO:H₂O gum fraction had the greatest concentration of EPA and DHA ($p < 0.05$). The 50:50 KO:H₂O ratio contained a lower concentration of EPA and DHA than the 75:25 KO:H₂O sample, but only the DHA content was statistically different ($p < 0.05$). There

were no differences ($p>0.05$) in fatty acid profiles in the oil fractions tested. The concentration of EPA and DHA in the fractions are consistent with others that reported the fatty acid profiles of the individual lipid classes. Araujo et al. (2014) separated neutral and polar lipids with different types of solvents, and the fatty acid profiles, based on % normalized mole units, showed similar results. The fatty acid composition of phospholipid in the krill oil was 28.5% EPA and 13.5% DHA whereas there was 9.7% EPA and 3.8% DHA in the triglycerides (Araujo et al., 2014). Other studies reported much lower amounts of EPA and DHA bound to triglycerides with ranges of 1-4% for each fatty acid (Castro-Gomez et al., 2015; Gigliotti et al., 2011; Xie et al., 2017). The greater EPA and DHA content in the oil fraction may be due to the diglycerides (DAG) separated into this fraction. Castro-Gomez et al. (2015) examined isolated fractions of krill oil and reported the DAG-rich fraction had 19.8% EPA and 6.1% DHA. The content and distribution of these PUFAs in krill oil are what make the product interesting. A phospholipid-rich fraction with a high content of ω -3 PUFAs can be a marketable food additive.

The 75:25 KO:H₂O oil and gum fractions contained different lipid classes and different types of fatty acids (Figure 2A). The fatty acid profile of the gum fraction presented with a greater concentration of ω -3 PUFAs ($p<0.05$) than the oil fraction. The gum fraction was composed of 55% ω -3 PUFAs while the oil fraction contained 26%. The gum fraction also had a lower ω -6/ ω -3 ratio when compared to the oil fraction. The ratios for oil and gum fractions are 0.65 and 0.16, respectively. Diets with a higher ω -6/ ω -3 ratio are a promoter of cardiovascular and inflammatory diseases (Simopoulos, 2002). Studies indicate that a lower level of ω -6 fatty acids in the blood decreases the occurrence of CVD (Lands, 2003). The recommended ratio of ω -6 to ω -3 fatty acids is 5:1, but the western diet is far below the optimal intake (Küllenberg et al., 2012). On the other hand, the oil fraction had a greater saturated fatty acid/unsaturated fatty acid

(SFA/UFA) ratio than the gum fractions of the 75:25 KO:H₂O samples (Figure 2B). The SFA/UFA ratios were 0.58 and 0.38 for the oil and gum fractions of the 75:25 KO:H₂O, respectively.

Identification and quantification of phosphatidylcholine species

After hydration and fractionation of the phospholipids, HPLC-MS was used to identify and quantify the phospholipid species that contained EPA and DHA in the gum fraction of the 75:25 KO:H₂O sample. Figure 3 shows a representative mass spectrum in the range where the PC species of interest are identified. Each PC molecule is identified with an arrow.

Concentration (µg/mL) and relative intensity data for each PC species is shown in Table 3. Results indicate the presence of PC20:5/20:5, PC20:5/22:6, and PC22:6/22:6 which are consistent with previous studies that found that the phosphatidylcholine species in krill oil has both EPA and DHA incorporated onto the same molecule (Castro-Gomez et al., 2015; Le Grandois et al., 2009; Winther et al., 2011). Typically, phospholipids contain a saturated and unsaturated fatty acid attached to sn1 and sn2; therefore, it is interesting for a PC species to contain two highly unsaturated fatty acids (Berdanier, 2008). It was not determined if the EPA or DHA was on the sn2 position; however, PC species with these ω-3 PUFAs on the sn2 position can increase cardiovascular health as well as brain function (Parmentier et al., 2007).

Of the three highly unsaturated phospholipids of interest, PC20:5/20:5 was the most abundant, followed by PC20:5/22:6 in the gum fraction of the 75:25 KO:H₂O sample (p<0.05). Results are consistent with Winther et al. (2011) who reported the same order of abundance with only slightly lower relative intensities for each of the three PC species of interest. A similar method of quantification was used in the previously mentioned study, but only relative intensity is reported.

Phospholipids have a different digestibility when compared to triglycerides. Due to phospholipids amphiphilic nature, they are not dependent on bile salts forming micelles for lipid digestion. Intestinal absorption of phospholipids is much higher than triglycerides as well. Up to 20% of dietary phospholipids can be absorbed passively in the intestine (Zierenberg and Grundy, 1982). Supplementation of phospholipids, especially ones containing EPA and DHA, will affect blood lipid profiles (Küllenberg et al., 2012). Incorporating these phospholipids double bound with ω -3 PUFAs into food or pharmaceutical products would enhance dietary intake of beneficial fatty acids. Lecithin, name for commercial phospholipids, have many functions such as forming liposomes, emulsion stability, and nutritional supplementation. Common sources of lecithin are soybeans and egg yolk, and are often byproducts of oil production. Soybean lecithin is comprised of about 45-60% linoleic acid, which is an essential ω -6 fatty acid (Stankova et al., 2013). Egg lecithin has more variation in the fatty acid profile, but still has a high proportion of saturated fatty acids and the ω -6 fatty acid linoleic acid. Phospholipids from krill oil could potentially be used in place of these lecithin types in food or pharmaceutical products depending on the function of the additive.

Conclusion

In conclusion, the results of this study indicate that water degumming principles applied to krill oil is a successful method to separate and concentrate phospholipids from other lipid classes. Along with the phospholipids, a majority of the ω -3 PUFAs found in krill oil were also concentrated in the gum fraction. Phospholipids found in krill oil contain an EPA and/or DHA on both the sn1 and sn2 position of a PC molecule. This approach may offer a simple technique to produce a lecithin product from krill oil that contain a healthier fatty acid profile than those

currently on the market. Future studies will focus on the functional properties of the phospholipids and their application in the food or pharmaceutical industries.

References

1. Akanbi, T. O., & Barrow, C. J. (2018). Compositional Information Useful for Authentication of Krill Oil and the Detection of Adulterants. *Food Analytical Methods*, 11(1):178-187.
2. Araujo, P., Zhu, H., Breivik, J. F., Hjelle, J. I., & Zeng, Y. (2014). Determination and structural elucidation of triacylglycerols in krill oil by chromatographic techniques. *Lipids*, 49(2):163-172.
3. Berdanier, C. D. Fatty acids and membrane function. Edited by Chow, C. K. *Fatty Acids in Foods and their Health Implications* (3rd edition). pp. 693-755. Boca Raton, FL. CRC Press Taylor & Francis Group.
4. Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, 37(8):911-917.
5. Braae, B. (1976). Degumming and refining practices in Europe. *Journal of American Oil Chemists' Society*. 43:353-357.
6. Bunea, R., El Farrah, K., Deutsch, L. (2004). Evaluation of the effects of Neptune krill oil on the clinical course of hyperlipidemia. *Alternative Medicine Review*. 9(4):420-428.
7. Castro-Gomez, M., Holgado, F., Rodriguez-Alacala, L., Montero, O., Fontecha, J. (2015). Comprehensive study of the lipid classes of krill oil by fractionation and identification of triglycerides, diacylglycerols, and phospholipid molecular species by using UPLC/QToF-MS. 8:2568-2580.
8. Ceci, L. N., Constenla, D. T., & Crapiste, G. H. (2008). Oil recovery and lecithin production using water degumming sludge of crude soybean oils. *Journal of the Science of Food and Agriculture*, 88(14): 2460-2466.
9. Dijkstra, A. J. (2018) Edible oil processing: introduction to degumming. *AOCS Lipid Library*. Retrieved April 4, 2018, from <http://lipidlibrary.aocs.org/OilsFats/content.cfm?ItemNumber=40325>
10. Dijkstra, A. J. & Segers J. C. (2007). Production and refining of oils and fats. Edited by Gunstone, F. D., Harwood, J. L., & Dijkstra, A. J. *The lipid handbook with CD-ROM*. (3rd edition). 143-262. Boca Raton, FL. CRC press.
11. Fritsche, K. L., & Johnston, P. U. (1990). Effect of dietary-linolenic acid on growth, metastasis, fatty acid profile and prostaglandin production of two murine mammary adenocarcinomas. *J. Nutr*, 120:1601-1609.
12. Gigliotti, J., Davenport, M.P., Beamer, S., Tou, J., Jaczynski, J. (2011). Extraction and characterization of lipids from Antarctic krill (*Euphausia superba*). *Food Chemistry*. 125(3):1028-1036.
13. Gilbert, H.F. (2000). *Basic concepts in biochemistry* (2nd edition). New York, NY. McGraw-Hill.
14. Kolakowska, A. (1991). The influence of sex and maturity stage of krill (*Euphausia superba* Dana) upon the content and composition of its lipids. *Polish Polar Research*. 12:73-78.
15. Küllenberg, D., Taylor, L. A., Schneider, M., & Massing, U. (2012). Health effects of dietary phospholipids. *Lipids in health and disease*, 11(1): 3.

16. Kutzner, L., Ostermann, A.I., Konrad, T., Riegel, D., Hellhake, S., Schuchardt, J.P., Schebb, N.H. (2016). Lipid class specific quantitative analysis of n-3 polyunsaturated fatty acids in food supplements. *Journal of Agriculture and Food Chemistry*. 65:139-147.
17. Lands, W. E. M. (2003). Diets could prevent many diseases. *Lipids*. 38(4):317-321.
18. Le Grandois, J., Marchioni, E., Zhao, M., Giuffrida, F., Ennahar, S., & Bindler, F. (2009). Investigation of natural phosphatidylcholine sources: separation and identification by liquid chromatography– electrospray ionization– tandem mass spectrometry (LC– ESI– MS2) of molecular species. *Journal of agricultural and food chemistry*, 57(14):6014-6020.
19. Lei, L., Ma, Y., Kodali, D. R., Liang, J., Davis, H. T. (2003). Ternary phase diagram of soybean phosphatidylcholine-water-soybean oil and its application to the water degumming process. *Journal of American Oil Chemists' Society*. 80:383-388.
20. Li, J., Wang, X., Zhang, T., Wang, C., Huang, Z., Luo, X., & Deng, Y. (2015). A review on phospholipids and their main applications in drug delivery systems. *Asian journal of pharmaceutical sciences*. 10(2): 81-98.
21. Mathews, C.K., van Holde, K.E., Ahern, K.G. (2000), *Biochemistry* (3rd edition). San Francisco, Ca. Addison Wesley Longman, Inc.
22. Nicol, S., James, A., Pitcher, G. (1987). A first record of daytime surface swarming by *Euphausia lucens* in the Southern Benguela region. *Marine Biology*. 94:7-10.
23. Parmentier, M., Mahmoud, C. A. S., Linder, M., & Fanni, J. (2007). Polar lipids: n-3 PUFA carriers for membranes and brain: nutritional interest and emerging processes. *Oléagineux, Corps gras, Lipides*, 14(3-4):224-229.
24. Saether, O., Ellingsen, T.E., Mohr, V. (1986) Lipids of North Atlantic krill. *Journal of Lipid Research*. 27:274-285.
25. Simopoulos, A. P. (2002). The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomedicine & pharmacotherapy*. 56(8):365-379.
26. Stankova, B., Kremmyda, L. S., Tvrzicka, E., & ŽÁK, A. (2013). Fatty Acid Composition of Commercially Available Nutrition Supplements. *Czech Journal of Food Science*, 31(3).
27. Winther B, Hoem N, Berge K, Reubsaet L (2010) Elucidation of phosphatidylcholine composition in krill oil extracted from *Euphausia superba*. *Lipids* 46:25-36.
28. Xie, D., Jin, J., Sun, J., Liang, L., Wang, Xia., Zhang, W., Wang, Xin., Jin, Q. (2017). Comparison of solvents for extraction of krill oil from krill meal: Lipid yield, phospholipid content, fatty acids composition and minor components. *Journal of Food Chemistry*. 2333:434-441.
29. Zierenberg O, Grundy SM. (1982). Intestinal absorption of polyenephosphatidylcholine in man. *J Lipid Res*. 23(8):1136–1142

Tables and Figures

Table 1: Relative distribution of lipid classes in respective fractions (% lipid class / sample or fraction) of initial krill oil and the 50:50 KO:H₂O and 75:25 KO:H₂O fractions separated by water degumming principles, determined by TLC-densitometry

	Lipid Class				
	TAG	FFA	CHOL	DAG	PHOS
100% Krill Oil	19.78±3.43 ^B	14.19±4.84 ^A	14.09±0.20 ^{AB}	5.63±0.89 ^B	46.28±0.32 ^B
50:50 KO:H₂O					
Oil	82.38±0.19 ^A	4.74±1.28 ^B	2.69±0.97 ^D	10.19±0.15 ^A	n.d.
Gum	23.88±6.36 ^B	14.51±3.28 ^A	11.87±0.55 ^C	n.d.	49.73±3.90 ^B
75:25 KO:H₂O					
Oil	80.67±3.70 ^A	4.58±0.29 ^B	2.97±0.55 ^D	11.78±2.57 ^A	n.d.
Gum	n.d.	15.54±1.52 ^A	16.84±0.87 ^A	n.d.	67.62±1.97 ^A

TAG- triglycerides, FFA- free fatty acids, CHOL- cholesterol, DAG- diglycerides, PHOS- phospholipids. n.d. Not detected.

^{A,B,C} Different letters mean significant differences within a column for a given class among fractions and ratios (p<0.05)

Table 2: Profile of major fatty acid composition (% fatty acid / sample or fraction) of initial krill oil and the 50:50 KO:H₂O and 75:25 KO:H₂O and fractions separated by water degumming principles.

	100% KO	50:50 KO:H₂O OIL	50:50 KO:H₂O GUM	75:25 KO:H₂O OIL	75:25 KO:H₂O GUM
C14:0	7.54±0.62 ^b	14.57±0.56 ^a	5.95±0.43 ^{bc}	14.21±0.86 ^a	4.21±1.22 ^c
C16:0	21.14±0.29 ^{abc}	20.50±0.37 ^{bc}	21.32±0.19 ^{ab}	20.05±0.37 ^c	21.67±0.70 ^a
C16:1 (C9)	3.84±0.14 ^b	6.98±0.18 ^a	3.16±0.14 ^b	6.78±0.49 ^a	2.10±0.54 ^c
C18:1 (C9)	8.42±0.20 ^b	13.22±0.19 ^a	7.43±0.29 ^b	12.56±0.61 ^a	5.82±0.75 ^c
C18:3 (C9,12,15)	4.38±0.09 ^b	5.14±0.12 ^a	4.20±0.01 ^b	5.17±0.13 ^a	3.85±0.15 ^c
C20:2 (C11,14)	8.91±0.06 ^b	14.05±0.19 ^a	7.65±0.15 ^c	13.94±0.30 ^a	5.88±0.73 ^d
C20:5 (EPA)	24.48±0.65 ^b	12.44±0.32 ^c	27.60±0.73 ^{ab}	14.12±2.01 ^c	30.98±2.34 ^a
C22:6 (DHA)	14.94±0.51 ^b	5.76±0.06 ^c	16.77±0.50 ^b	6.47±0.90 ^c	19.82±1.69 ^a

^{a,b,c} Different letters mean significant differences within a row for a given fatty acid among fractions and ratios (p<0.05).

Table 3: Identified phosphatidylcholine (PC) species containing EPA (20:5) and DHA (22:6) with the most abundant PC species by mass of molecule.

75:25 KO:H₂O gum fraction			
	Mass (m/z)	Relative Intensity	Concentration (µg/mL)
PC20:5/20:5	780.56	45.27±1.07	54.77±2.90
PC20:5/22:6	828.56	40.41±0.86	48.98±4.31
PC22:6/22:6	852.56	12.63±0.08	15.30±1.08
PC16:0/20:5	878.57	100	121.12±8.71

Mass of the PC species is expressed in Daltons. Data are expressed as mean± standard deviation for phosphatidylcholine species relative intensity and concentration. Concentration is expressed as µg/mL sample

Figure 1. Thin layer chromatography displaying lipid classes for initial krill oil and the 25:75 KO:H₂O, 50:50 KO:H₂O, and 75:25 KO:H₂O and fractions separated by water degumming principles as well as standards used for identification. Each number represents a separate lane. 1- mono-, di-, triglyceride standard, 2- free fatty acid (oleic acid) standard, 3- cholesterol standard, 4- phospholipid standard, 5- 100% KO, 6- 25:75 KO:H₂O, 7- 50:50 KO:H₂O oil fraction, 8- 50:50 KO:H₂O gum fraction, 9- 75:25 KO:H₂O oil fraction, 10- 75:25 KO:H₂O gum fraction. TAG- triglyceride, FFA- free fatty acid, CHOL- cholesterol, DAG- diglycerides, PHOS- phospholipids.

Figure 2. Types of fatty acids found in krill oil and oil and gum fractions of the 75:25 KO:H₂O ratio trial. (A) Comparison of SFA (saturated fatty acids), UFA (unsaturated fatty acids), total $\omega 3$ (omega-3 fatty acids), and total $\omega 6$ (omega-6) fatty acids found in each sample. 100% KO is included as a reference for the fatty acid comparison. Data are presented as % fatty acid in total fatty acids in the fraction. (B) Comparison of SFA/UFA and $\omega 6/\omega 3$ ratios based on fatty acid content of krill oil and separated 75:25 KO:H₂O oil and gum fractions. a,b,c Different letters indicate statistical differences ($p < 0.05$)

Figure 3. Representative mass spectrum of 75:25 KO:H₂O ratio gum fraction. PC species of interest were identified by m/z. Each are labeled in figure. Relative intensities are shown on the y-axis with the mass on the x-axis.

PART TWO: Functional properties of a concentrated egg yolk protein powder using a one-step organic solvent extraction process

CHAPTER IV

REVIEW OF LITERATURE

Introduction

Egg yolk is an inexpensive and excellent source of high quality protein, lipid, vitamins, and minerals. The composition of dried egg yolk product is 59% lipid, 34% protein, 1% carbohydrate, and 4% ash (USDA, 2016). Breaking the egg yolk into parts offers many functional properties. Phospholipids, called lecithin in industry, are extracted from the egg yolk to be used as an emulsifying agent in food and pharmaceutical products. Lipids are often removed from egg yolk by organic solvent extraction methods. By removing lipid, the protein concentration of the egg yolk is increased, and therefore has the potential for other uses; however, the functionality may be altered due to protein denaturation by organic solvents. Therefore, the aim of this review was to understand the composition of egg yolk proteins and evaluate studies that tested the functionality of egg yolk protein concentrates after organic solvent lipid extraction.

Organic solvent lipid extraction

Lipid extraction is commonly performed by employing organic solvents such as chloroform, methanol, ethanol, hexane, and isopropanol to solubilize and separate the lipids from other components of a sample. Solvents range from extremely polar, such as methanol, to those completely nonpolar like petroleum ether. Chloroform-methanol (CM) is a common solvent combination used in laboratory settings for lipid extraction, but they are not food grade solvents.

Hexane-isopropanol (HI) is a combination of food grade solvents that are frequently used as an alternative to CM. Both of these solvent mixtures employ a nonpolar and polar component to ensure the removal of neutral and polar lipid classes. Hexane-isopropanol is a typical solvent combination used in the extraction of lipids from egg yolk (Ahn et al., 2006). Methyl-tert-butyl-ether (MTBE) is an alkyl ether that is more polar than hydrocarbons, but not as polar as alcohols. It has been used as an alternative to CM because lipids are contained in the upper layer rather than the bottom layer formed when CM is used (Matyash et al., 2008). This allows the lipid to be easily removed without contamination of other components of a sample.

Several lipid extraction methods have been developed over the years such as Folch, Bligh and Dyer, and Soxhlet to fit needs of researchers or to be more efficient. Traditionally, these methods call for the use of chemicals that are not food grade, are time consuming, or more labor intensive (Xie et al., 2017). For these reasons other methods that utilize safer organic solvents and require less work, such as one-step extraction methods, have been researched and developed. One-step lipid extraction methods involve mixing a set ratio of an organic solvent with a sample and centrifugation to collect lipid rich solvent fraction.

Rose and colleagues (2019) studied the extraction efficiency and compared lipid classes and fatty acid profiles of insect, egg yolk, and krill powders obtained from a newly developed one-step extraction method using different solvents. The researchers employed a 1:10 sample to solvent ratio using hexane, hexane-isopropanol (3:2, v:v), chloroform, chloroform-methanol (2:1, v:v), and methyl-tert-butyl ether (MTBE) as the organic solvents. In egg yolk powder CM, HI, and MTBE had low but comparable lipid extraction efficiencies (24-31%) while extracting more phospholipids and cholesterol than other solvents (Rose et al., 2019). Following extraction, the

residual defatted sample has an increased protein concentration which may have potential as a food source or functional additive (L'Hocine et al., 2006).

Composition and functional properties of egg yolk

Low density lipoproteins (LDL) and high density lipoproteins (HDL) make up a majority of the egg yolk protein profile; 65% and 16%, respectively (Mine, 2008). HDLs are found exclusively in the granule fraction (70%) (Anton et al., 1997). LDL is the main protein component (85%) of the plasma fraction (McCully et al., 1962). Functional properties of egg yolk are dependent on the components. LDL is considered “soluble” due to the low density and not precipitating when centrifuged (Anton et al., 1997). HDL molecules, found in granules, are relatively water insoluble due to their compact structure (Kaufman, 2017). Salt is added to dissociate granules to increase solubilization of yolk proteins (Le Denmat et al., 2000). Dissociation of granular HDL affects gelation of egg yolk proteins, and adding salt to a protein dispersion promoted a stronger gel (Li et al., 2017). Nakamura et al. (1982) reported that LDL is an important component for egg yolk gel formation. This is attributed to LDL high sensitivity to heat and denaturing at lower temperatures (Kiosseoglou and Paraskevopoulou, 2005). HDL structure causes it to be more heat resistant than LDL of plasma (Anton, 2013; Valverde et al., 2016). LDL gels were more stable in a range of environmental conditions when compared to ovalbumin and bovine serum albumin gels (Nakamura et al., 1982).

Valverde and others (2016) worked to develop a new type of gel prepared with egg yolk and its respective plasma and granules. Rheological tests showed the granule was more resistant to heating than the plasma mixture. Yolk and plasma gels had similar values for hardness and deformation work for both compression percentages, but granule gels were not as strong or hard. Microstructure of granule gels appeared more rough and corresponded to protein complexes,

while plasma gels had a smooth microstructure. Granule gels exhibited lower values of redness and yellowness than yolk and plasma gels which was attributed to the lower lipid content of the fraction. The egg yolk fractions were also used to simulate future applications for the product, and low-caloric or high protein desserts or gels were prepared.

Livetins are water soluble proteins that account for about 9% of egg yolk protein, and work done by Chalamaiah and others (2017) aimed to study the physiochemical and functional properties of the proteins. The livetin fraction of egg yolk contains immunoglobulin Y. It has been recognized as a source of antibodies and used as an alternative to antibiotics in animal feed (Kaufman, 2017). The isoelectric point of the proteins was at pH 5.7. Solubility of the livetins was greater than 86% over a wide pH range with the highest at pH 2 and 12. Results of the study indicate that individual protein types found in egg yolk, livetins, exhibit desirable physiochemical and functional properties (Chalamaiah et al., 2017).

Functional properties of lipid extracted food products

Egg yolk. Extracting lipid from egg yolk using a one-step method could offer a time efficient method to produce egg yolk phospholipids for use as functional food. Employing a combination of polar and non-polar solvents are useful for extracting lipids from egg yolk because the polar solvents denature proteins, such as LDL, to allow the nonpolar solvent to dissolve lipids held inside (Kovalcuks and Duma, 2014). This alteration of the structure could affect the functionality of the protein. When lipid is extracted from egg yolk dispersions, the polar solvent interacts with hydrophilic side chains and causes the hydrophobic parts to be exposed to the organic solvents (Khmelnitsky et al., 1991). Methanol acts liquid phase and causes a destruction of the hydration shell, which is likely the reason proteins denature when exposed to polar solvents (Khmelnitsky et al., 1991). Polar solvents cause disruptions in

hydrogen bonding and electrostatic interactions in the protein that cause them to aggregate and precipitate (Kovalcuks and Duma, 2014; Lapanje, 1978).

Chung and Ferrier (1991) aimed to determine the effect of solvents on emulsifying properties and water solubility of partially defatted egg yolk. Lipid from egg yolk powder was extracted by using a vacuum system to suction solvent through sample after mixing a ratio of 1:2 powder to solvent. Solvents used included hexane, chloroform-methanol (2:1, v:v), isopropanol, hexane-isopropanol (77:23, w:w), ethanol (95%), or hexane-ethanol (77:23, w:w). Up to four extractions were applied to samples extracted with hexane and chloroform-methanol while only two were completed for the other solvent extractions. Proximate composition, water solubility, and emulsifying activity of the defatted powder. Results were compared to a control sample of egg yolk powder with no extractions. SDS-PAGE and Western blot were performed to separate and analyze soluble proteins from each solvent extraction.

Extraction with hexane-isopropanol (77:23, w:w) was shown to remove the most lipid from the egg yolk powder that resulted in a proximate composition of $60.4 \pm 0.92\%$ protein and $29.7 \pm 0.36\%$ lipid (Chung and Ferrier, 1991). The egg yolk protein concentrates from all solvents resulted in lower emulsifying activity as well as water solubility. Samples from the chloroform-methanol extractions displayed about 41% less emulsifying activity while hexane and hexane-isopropanol showed the closest results when compared to a control but both were significantly less ($p < 0.05$) (Chung and Ferrier, 1991). Chloroform-methanol and ethanol produced a protein concentrate where the protein solubility decreased from 15.7% to 3.2% and 5%, respectively. Hexane, isopropanol, and hexane-isopropanol produced the most comparable water solubility to the control, but was still significantly lower ($p < 0.05$) (Chung and Ferrier, 1991). Multiple extractions with chloroform-methanol resulted in reduced or broader bands of water soluble

proteins which indicated the solvent denatured proteins and caused them to become less water soluble. Overall, hexane and isopropanol effected the functional properties the least (Chung and Ferrier, 1991).

In order to remove cholesterol from egg yolk, Paraskevopoulou and Kiosseoglou (1994) studied the influence of petroleum ether and petroleum ether-ethanol (35:65 v/v) on emulsion stability, rheological properties of a mayonnaise-like emulsion, and foaming properties of extracted egg yolk. Lipid extraction was performed by mixing dried egg yolk with either petroleum ether or petroleum ether-ethanol (35:65 v/v) in a 1:10 sample to solvent ratio for 2.5 hr. Solvent and lipid were removed by filtering under vacuum, and powder was re-washed with 200 mL of solvent. Dry matter, lipid, cholesterol, and lipid phosphorous (phospholipid) content were determined for the liquid, dried, and extracted egg yolk. There were no statistical analyses conducted on the results of this study; however, results of the study indicate egg yolk extracted with petroleum ether-ethanol also showed the greatest lipid reduction. Petroleum ether-ethanol extracted egg yolk had the least stable oil/water emulsion and also did not form a mayonnaise-like emulsion which was attributed to the removal of phospholipids. Foam expansion and stability was higher for the extracted egg yolk than the initial dried yolk, which was associated with the reduction of lipid in the sample. However, the petroleum ether extracted yolk had higher expansion and stability results than the petroleum ether-ethanol extracted yolk. This indicated ethanol, the polar solvent, may have had a negative effect on the protein concentrate even though it extracted more lipid. These results indicated that solvent extractions did lower the cholesterol content, but solvent mixtures such as petroleum ether-ethanol have a negative influence on functional properties of the egg yolk protein concentrate (Paraskevopoulou and Kiosseoglou, 1994).

The previous authors published another study using a similar concept of extracting lipid and cholesterol from egg yolk in order to test the influence of the extraction on gelling properties and cake structure (Paraskevopoulou and Kiosseoglou, 1997). In addition to methodology employing petroleum ether and petroleum ether-ethanol (35:65 v/v), the authors extracted egg yolk with 20:80 ethanol-water containing 1.5% polysorbate 80 and supercritical CO₂ (SC-CO₂). Gels were prepared by mixing dispersions of 25, 30, and 35% (w/v) of yolk solids. Texture profile analysis (hardness, cohesiveness, and springiness) was determined for each gel type by a double compression test. No statistical analyses were conducted; however, results indicated that petroleum ether and petroleum ether-ethanol (35:65 v/v) extractions increased the concentration of protein and resulted in the hardest gels. This was attributed to higher protein and low-density lipoproteins (LDL) concentrations (Paraskevopoulou and Kiosseoglou, 1997). Gels prepared with yolk extracted with the ethanol-water mixture were less hard than the petroleum extracts, but similar to control gels. Authors noted this was due to the reduction of water soluble proteins, and an increase in HDL concentration (Paraskevopoulou and Kiosseoglou, 1997). Gels prepared with the extracted egg yolks generally were less cohesive. The springiness of the gels exhibited the same trend of hardness with the extracted egg yolk gels were generally springier than the control. However, gels from the ethanol-water mixture showed the opposite trend with cohesiveness and springiness which could be due to the types of protein present. The prepared cakes showed a trend of being harder and less cohesive and springy than the control cake. Results of the study are accredited to the differences in protein and lipid content of the egg yolk used to prepare the gels and cakes (Paraskevopoulou and Kiosseoglou, 1997).

Soybean protein isolates. The effects of alternative defatting and lipid extraction methods on the composition and functional properties of soybean protein isolates were examined

by L'Hocine and others (2006). Soybean meal was defatted by mixing meal with solvent in a 1:1 to 1:3 ratio for 30 min and the solvent decanted. The extraction process was repeated 2 more times. The protein isolates had a protein content greater than 92% for all extraction solvents. Ethanol and methanol extractions showed alterations to protein structures which was indicated by thermal properties of the soy protein isolates. The defatting techniques did not affect the solubility or dispersability of the protein isolates. The defatted protein isolates showed better emulsion activity. This was attributed to the organic solvent denaturing proteins and increasing surface hydrophobicity (Rickert and others, 2004). In conclusion, the results show that solvent extraction increases protein content of soy protein isolates, and their functionalities can be positively or negatively affected by the solvent. Also, methanol is a poor choice as a solvent for lipid extraction when looking at protein functionality.

The aim of this review was to investigate previous research on the functional properties of egg yolk components and the effects of various organic solvents used for lipid extraction on the defatted egg yolk protein functionality. Organic solvent lipid extraction is an effective method to remove cholesterol and other lipids while increasing the protein content of egg yolk powder. Impact of lipid extraction on the functional properties is influenced by organic solvent used for the extraction. Further research is needed on one-step extractions using other food grade organic solvents as well as a more comprehensive functionality examination.

References

1. Ahn D.U., Lee S.H., Singam H., Lee E.J., Kim J.C. (2006) Sequential separation of main components from chicken egg yolk. *Food Science and Biotechnology*. 15(2):189–195.
2. Anton, M., & Gandemer, G. (1997). Composition, solubility and emulsifying properties of granules and plasma of egg yolk. *Journal of Food Science*. 62(3):484-487.
3. Anton, M. (2013). Egg yolk: structures, functionalities, and processes. *Journal of Science Food Agriculture*. 93:2871-2880.
4. Chalamaiah, M., Esparza, Y., Temelli, F., & Wu, J. (2017). Physicochemical and functional properties of livetins fraction from hen egg yolk. *Food Bioscience*. 18:38-45.
5. Chung, S.L. and Ferrier, L.K. (1991). Partial lipid extraction of egg yolk powder: effect on emulsifying properties and soluble protein fraction. *Journal of Food Science*. 56(5):1255-1258.
6. Kaufman, I. (2017) The recovery of protein from egg yolk protein extraction granule byproduct. Master of Science in Agriculture. California Polytechnic State University.
7. Kiosseoglou, V. and Paraskevopoulou, A. (2005). Molecular interactions in gels prepared with egg yolk and its fractions. *Food Hydrocolloids*. 19(3):527-532.
8. Khmelnitsky, Y. L., Belova, A. B., Levashov, A. V., & Mozhaev, V. V. (1991). Relationship between surface hydrophilicity of a protein and its stability against denaturation by organic solvents. *FEBS letters* 284(2):267-269.
9. Kovalcuks, A. and Duma, M. (2014). Solvent extraction of egg oil from liquid egg yolk. 9th Baltic Conference on Food Science and Technology- Food for Consumer Well-Being: Foodbalt 2014:253-256.
10. Lapanje, S. (1978). *Physicochemical aspects of protein denaturation*. John Wiley & Sons, New York, 142
11. Le Denmat, M., Anton, M., Beaumal, V. (2000). Characterization of emulsion properties and of interface composition in O/W emulsions prepared with hen egg yolk, plasma and granules. *Food Hydrocolloids*, 14 (6):539-549.
12. L'Hocine, L., Boye, J. I., Arcand, Y. (2006). Composition and functional properties of soy protein isolates prepared using alternative defatting and extraction procedures. *Journal of food science*. 71(3):C137-C145.
13. Li, J., Li, X., Wang, C., Zhang, M., Xu, Y., Zhou, B., Su, Y., Yang, Y. (2017). Characteristics of gelling and water holding properties of hen egg white/yolk gel with NaCl addition. *Food Hydrocolloids*. 77:887-893.
14. Matyash, V., Liebisch, G., Kurzchalia, T. V., Shevchenko, A., Schwudke, D. (2008). Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. *Journal of lipid research*. 49(5):1137-1146.
15. McCully, K. A., Mok, C. C., Common, R. H. (1962). Paper electrophoretic characterization of proteins and lipoproteins of hen's egg yolk. *Canadian Journal of Biochemistry and Physiology*. 40(7):937-952.
16. Mine, Y. 2008. *Egg bioscience and biotechnology*. Wiley-Interscience, Hoboken, NJ.
17. Nakamura, R., Fukano, T., Taniguchi, M. (1982). Heat-induced gelation of hen's egg yolk low density lipoprotein (LDL) dispersion. *Journal of Food Science*. 47(5):1449-1453.

18. Paraskevopoulou, A. and Kiosseoglou, V. (1994). Cholesterol and other lipid extraction from egg yolk using organic solvents: effects on functional properties of yolk. *Journal of Food Science*. 59(4): 766-768.
19. Paraskevopoulou, A. and Kiosseoglou, V. (1997). Texture profile analysis of heat-formed gels and cakes prepared with low cholesterol egg yolk concentrates. *Journal of Food Science*. 62(1): 208-211.
20. Rickert, D. A., Johnson, L. A., Murphy, P. A. 2004. Functional properties of improved glycinin and beta-conglycinin fractions. *Journal of Food Science*. 69(4):C303–311.
21. Rose, A., Jaczynski, J., Mo Ku, K. Chiu Y., Matak, K. (2019). Defatting insect powders to enhance protein quality and concentration. Characterization of lipids and the protein co-products from various food sources using a one-step organic solvent extraction process. Master's thesis, West Virginia University.
22. USDA National Nutrient Database for Standard Reference, Release 28. (2016) US Dept of Ag Research Services, Nutrient Data Laboratory. <https://ndb.nal.usda.gov/ndb/>
23. Valverde, D., Laca, A., Nel Estrada, L., Paredes, B., Rendueles, M., and Diaz, M. (2016). Egg yolk and egg yolk fractions as key ingredient for the development of a new type of gels. *International Journal of Gastronomy and Food Science*. 3:30-37.
24. Xie, D., Jin, J., Sun, J., Liang, L., Wang, X., Zhang, W., Jin, Q. (2017). Comparison of solvents for extraction of krill oil from krill meal: Lipid yield, phospholipids content, fatty acids composition and minor components. *Food chemistry*. 233:434-441.

CHAPTER V

Functional properties of a concentrated egg yolk protein powder using a one-step organic solvent extraction process

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Abstract

Egg yolk is a functional food product that contains high quality protein and lipids, and has emulsifying and gelation capabilities. The lipid is often extracted using organic solvents, but few studies look at the effect of specific organic solvents, especially MTBE, on the functionality of the remaining protein concentrate. Therefore, the objective of this study was to determine how different organic solvents would affect proximate composition, water solubility, and gelling of egg yolk protein concentrate recovered when lipid was extracted from spray-dried egg yolk using a novel one-step organic solvent extraction method. Organic solvents tested were hexane-isopropanol (HI) (3:2, v:v), chloroform-methanol (CM) (2:1, v:v), and methyl-tert-butyl ether (MTBE). Egg yolk protein concentrate contained a greater concentration of protein, ranging from 50-66%, compared to initial egg yolk powder with 36% ($p<0.05$). Lipid was also reduced in each protein concentrate and ranged from 21-25% versus 49% in the initial egg yolk powder ($p<0.05$). Solubility of the initial egg yolk powder and protein concentrates was consistent among all samples obtained when HI and MTBE were used as the extraction solvent ($p>0.05$). Solubility decreased from 20% to 4% protein ($p<0.05$) when CM was used, likely the polarity of the methanol denatured proteins and reduced functionality. Gels from MTBE and HI protein concentrates had similar hardness ($p>0.05$), and were harder than initial egg yolk powder gels ($p<0.05$). MTBE gels were springier, more cohesive, and gummier ($p<0.05$) with similar resistance to initial egg yolk powder gels ($p>0.05$). The gels made from protein concentrates were less yellow ($p<0.05$) than initial gels because fat soluble pigments were removed with lipid extraction. The results of this study show that the functionality of the protein concentrate was best retained when MTBE was used as the organic solvent in the lipid extraction process.

KEYWORDS: protein concentrate, functional properties, organic solvent, lipid extraction

Introduction

Egg yolk contains high quality nutrients such as protein, fatty acids, vitamins, and minerals. The composition of dried egg yolk product is 59% lipid, 34% protein, 1% carbohydrate, and 4% ash (USDA, 2016). Egg yolk proteins consist of mainly high and low-density lipoproteins with others such as livetins, lipovitellins, and phosvitin. Lipids in egg yolks are found exclusively associated with lipoproteins (Anton, 2007). Phospholipids, specifically phosphatidylcholine from egg yolk, often contain omega-3 fatty acids and are added to infant formulas as a nutritive additive. In addition to its nutritional components, egg yolk exhibits many functional properties, like emulsification and gelation, and is a common ingredient in foods such as mayonnaise and dressings. Low-density lipoproteins (LDL) proteins are important for gel formation, and are stable in a wide range of environmental conditions (Nakamura et al., 1982). This is accomplished by unfolded proteins forming networks by disulfide bonds and hydrophobic interactions (Alleoni, 2006). Phospholipids extracted from egg yolk also offer many functionalities in the pharmaceutical industry; however, the remaining concentrated protein is often discarded.

Employing a combination of polar and non-polar solvents are useful for extracting lipids from egg yolk. Polar solvents will denature lipoproteins by disrupting the hydrophilic side chains which then lead to the exposure of the hydrophobic regions to the non-polar solvents that dissolve the lipids held inside (Khmelnitsky et al., 1991 and Kovalcuks and Duma, 2014). This alteration of structure could affect the functionality of the protein and cause them to aggregate and precipitate (Lapanje, 1978). It has been shown that organic solvents decrease certain egg yolk protein functional properties while also increasing others depending on solvent types and methodology performed. Studies have used chloroform, methanol, hexane, isopropanol,

petroleum ether, ethanol to remove lipid from egg yolk to determine if it altered the functionality of the protein (Larsen and Fronning, 1981; Chung and Ferrier, 1991; Paraskevopoulou and Kiosseoglou, 1994, 1997). Solvents range from extremely polar, such as methanol, to those completely nonpolar, like petroleum ether. The polar solvent ethanol is used to extract the phospholipids for a functional ingredient; however, it greatly decreases function properties of proteins compared to their native state (Marcet et al., 2017). Chloroform-methanol (CM) is a common solvent combination used in lab settings for lipid extraction, but they are not food grade solvents. Hexane-isopropanol (HI) is a combination of food grade solvents that are frequently used as an alternative to CM. Both solvent mixtures employ a nonpolar and polar component to ensure the removal of neutral and polar lipid classes. Methyl-tert-butyl ether (MTBE) is an alkyl ether that has polar tendencies, but is less polar than alcohols. Rose et al. (2019a) studied the efficiency of a one-step lipid extraction method and found that CM (2:1, v:v), HI (3:2, v:v), and MTBE had similar lipid extraction efficiencies (31%, 27%, and 24%, respectively) and similar lipid and fatty acid profiles to the original egg yolk powders.

The effects of these specific organic solvents on the functionality of resulting protein concentrates have not been studied; therefore, the objective of this study was to determine if a one-step lipid extraction using various organic solvents affects the functional properties such as water solubility and gelling of the concentrated egg yolk proteins.

Materials and Methods

Spray dried egg yolk powder was purchased from Magic Flavors (Seattle, Washington) and stored at -80°C until analyses were conducted.

Concentration of protein through lipid extraction

Lipid extraction was performed as described by Rose et al. (2019a). Hexane-isopropanol (HI) (3:2, v:v), chloroform-methanol (CM) (2:1, v:v), and methyl-tert-butyl ether (MTBE) were used as the extraction solvents based on lipid extraction efficiencies and extraction of all lipid classes (Rose et al., 2019a). CM solvent combination, not food grade, is commonly used in research settings and was tested against food grade HI and MTBE solvents. Three grams of powdered egg yolk were weighed into a 35mL Teflon-lined Pyrex glass test tube, and 30mL of the appropriate solvent was added. The sample and solvent mixture was vortexed for 60 sec and then transferred to a 250 mL beaker with a proportional magnetic stir bar. The sample was mixed for 15 min on a stir plate at 300 rpm. The mixture was transferred back to the test tube and centrifuged at 900 x g at 10°C for 10 min. The solvent containing the lipids was discarded and the residual concentrated protein was dried overnight under the hood. Extractions were replicated three times for each solvent and yield was calculated using the following formula;

$$\text{Yield (\%)} = \frac{\text{weight before extraction}}{\text{residual powder weight}} \times 100$$

Egg protein concentrates from each solvent extraction was mixed to make a bulk homogenate sample for each CM, HI, and MTBE. Proximate composition was conducted to verify composition.

Proximate composition

Samples from each solvent extraction (protein concentrates) and initial egg yolk powder were analyzed for proximate composition (moisture, ash, crude protein, and lipid). All procedures followed AOAC (1995) approved methods. Briefly, moisture content of the samples was determined using the oven drying method. Samples measuring 0.5 grams were placed in a

100°C oven overnight. The following formula was used to determine moisture content (ASTM, 1993);

$$\text{Moisture content (MC)} = \frac{\text{initial weight} - \text{oven dried weight}}{\text{initial weight}} \times 100 \text{ of total mass}$$

Samples that had been previously dried for the moisture content analysis were ashed in an A1500 furnace (F-A1525M-1; Thermolyne Corporation; Dubuque, Iowa) muffle oven overnight at 550°C. The ash content was determined by the following formula (ASTM, 1993);

$$\text{Ash content (AC)} = \frac{\text{dried weight}}{\text{initial weight}} \times 100 \text{ of total mass}$$

Crude protein was measured by Kjeldahl N assay using the Kjeltec 2300 (Foss North America; Eden Prairie, Minnesota). Steps of the assay included digestion, distillation, and titration. 0.2 grams of sample and Kjel-tabs were dissolved in 10mL of sulfuric acid. Samples were digested by heating at 400°C for 50 min with constant vacuum by exhaust manifold. After digestion, 25 mL of distilled water was added, and the solution was titrated. The amount of acid (mL) required to titrate the solution was used to determine nitrogen content in moles. Moles of nitrogen were multiplied by 14.01 (N atomic number) to get grams of nitrogen, and thereby crude protein was calculated grams of nitrogen by 6.25.

$$\text{Crude protein (CP)} = \frac{0.1 \text{ N HCl} \times 14.01 \times 6.25 \times \text{mLs acid}}{\text{sample weight} \times 1000} \times 100 \text{ of total mass}$$

Lipid content was measured by petroleum ether in a soxhlet extractor where 0.5 grams of sample was folded in a Whatman 41 filter paper. Following 18 h of extraction, samples were dried overnight at 100°C. Fat content, on a dry matter basis, was determined with the following formula (AOAC, 1995);

$$\text{Lipid content} = \frac{(\text{initial weight} - \text{dried weight}) - \text{grams moisture}}{\text{initial weight}} \times 100 \text{ of total mass}$$

Water soluble protein

Initial egg yolk powder and protein concentrates were analyzed for water soluble protein content. Two grams of sample was reconstituted with 20 mL of distilled water. The mixture was stirred for 30 min at room temperature, and transferred to a 35 mL test tube. Then tubes were centrifuged at room temperature for 30 min at 5,000 x g to separate water soluble and insoluble proteins. The supernatant was decanted into freeze-dry cups, and both the water soluble and insoluble fractions were freeze-dried (VirTis Freeze Dryer, SP Scientific, Stone Ridge, NY, USA) to remove water. Protein content of each was calculated by Kjeldahl assay previously mentioned. Protein solubility was calculated by the following formula

$$\% \text{ soluble protein} = \frac{\text{protein content of water soluble fraction (grams)}}{\text{protein in sample (grams)}} \times 100$$

The results are reported in a dry matter basis. Protein in grams was determined by multiplying percent protein, determined by Kjeldahl, by weight of initial sample and freeze-dried water-soluble fraction.

Gelling properties

The gelling properties of the initial egg yolk powder and protein concentrates were measured by reconstituting the powders to a moisture content of 80% (w/w) with 0.5 M NaCl salt solution in distilled water. The salt solution was used to increase solubility and denature proteins to form a gel (Anton, 2013). Gels would not form using distilled water alone. The reconstituted powders were poured into lightly oiled 50 mL plastic tubes and capped. Samples were cooked in a 90°C water bath for 20 min, and then immediately cooled on ice to room

temperature. Gels were stored in tubes overnight at 4°C. Six tubes of each gel type were prepared for analyses.

Texture profile analysis

Previously cooked gels were removed from tubes and cut into cylinders measuring 2.54 in both height and diameter. Texture profile analysis (TPA) of the prepared gels was conducted using a 70 mm-diameter plate probe mounted to the TA-HDi Texture Analyzer (Texture Technologies Corp.; Scarsdale, NY). Samples were subjected to a double compression test (25% compression) with a 50kg load cell and a speed of 1mm/s. The maximum force (g) observed during compression was used to analyze hardness, springiness, cohesiveness, gumminess, and resilience. TPA was analyzed by Texture Expert software. At least six gel samples were tested per solvent type.

Water holding capacity

Water holding capacity (WHC) of gels measuring 2.54 in both height and diameter, was analyzed by a uniaxial compression test using 70 mm-diameter plate probe mounted to the TA-HDi Texture Analyzer (Texture Technologies Corp.; Scarsdale, NY) with a 50kg load cell and a speed of 1mm/s. Gels were placed between two dry Whatman G08 filter papers, and double compressed to 50%. Water holding capacity was determined by the following equation;

$$\text{WHC (\%)} = \frac{\text{initial water content (g)} - \text{water lost (g)}}{\text{initial water content (g)}} \times 100$$

Initial water content was determined by moisture analysis with previously mentioned methods. Water loss was determined by weighing filter paper before and after compressions, and taking the difference. At least six gel samples were tested per solvent type.

Color measurements

Color analysis of gels from each sample type were measured using a colorimeter (Minolta Camera Co. Ltd, Osaka, Japan) calibrated with a standard white plate. Whiteness was calculated after measuring L^* (lightness: 0-100), a^* (intensity of red color: -60 to +60), and b^* (intensity of yellow color: -60 to +60).

$$\text{Whiteness} = 100 - [(100-L)^2 + a^2 + b^2]^{1/2}$$

At least six color measurements of gels were taken per solvent type.

Statistical analysis

Lipid extractions were replicated three times for each solvent used in the methodology. Proximate composition of the protein concentrates was completed in duplicates for each of the extraction replicates and compared to the initial egg yolk powder. Functional properties of water solubility and gelling (texture profile analysis, water holding capacity, and color) were all replicated at least three times and compared to initial egg yolk powder. Results are reported as mean $\pm$ standard deviation (SD). Statistical analyses were conducted using SAS JMP version 13 (SAS Institute Inc., North Carolina, USA). One-way independent measures analysis of variance (ANOVA) was used to determine individual differences between treatments. Post-hoc analysis was conducted using Tukey-Kramer's HSD test with a significance level of ($P < 0.05$).

Results and Discussion

Yield and Proximate composition

The recovery yield of defatted egg yolk by the one-step organic solvent lipid extraction ranged from 46-57% with statistically greater yields when HI and MTBE were used as the organic solvent (Figure 1; $p < 0.05$). In addition, when HI and MTBE were used as the organic

solvents the protein concentrate was similar to the powdery texture of the initial egg yolk powder; on the other hand, when CM was used as the organic solvent the resulting protein concentrate was hard with a grainy texture, consistent to the description by Chung and Ferrier (1991). Polar solvents such as methanol interact with hydrophilic side chains and cause the hydrophobic regions to be exposed to the organic solvents (Khmelnitsky et al., 1991; Kovalcuks and Duma, 2014). This exposure causes proteins to aggregate and precipitate which could explain the texture of the CM defatted egg yolk (Lapanje, 1978).

The proximate composition of the initial powder on a dry matter basis was 49% lipid, 36% protein, and 6% ash. Proximate composition of the defatted egg yolk revealed that protein was concentrated in all samples with concentrations from 57-66% protein compared to 36% protein in the initial egg yolk powder ($p < 0.05$). Extraction by MTBE and CM demonstrated similar reduction in lipid content ($p > 0.05$) and both extracted more lipid than HI ($p < 0.05$). Even though it had the lowest yield, the use of 2:1 CM showed the greatest concentration of protein (66%) ($p < 0.05$), while 3:2 HI and MTBE showed similar protein concentrations (57-58%). It is likely that the methanol caused proteins to precipitate from exposure to the highly polar solvent. One of the roles of the polar solvent in lipid extraction is to denature proteins like those in egg yolk to allow the nonpolar solvent to extract neutral lipids. Membrane proteins of LDL may have been caught in the organic solvent phase during extraction with the less polar solvents of MTBE and HI.

Water solubility

Solubility of the concentrated egg yolk protein and initial powder were measured and results are expressed as percent soluble protein and shown in Figure 2. Protein solubility was not altered in powders extracted using HI and MTBE ($p > 0.05$). This is consistent with Rose and

others (2019b) who measured solubility of protein concentrates extracted from cricket or locust powders. They found that when MTBE was used as the organic solvent in the lipid extraction process, there were no significant differences in solubility on a pH gradient that ranged from pH 5.0- pH 11.0 (Rose et al., 2019b). On the other hand, in this current study when CM was used as the extraction solvent for egg yolk lipids, the solubility of the concentrated protein was significantly reduced from 20% to 4% ($p < 0.05$). The polar solvent interrupts hydrogen bonding and electrostatic interaction in the protein which could in turn alter the equilibrium of the hydrophilic and hydrophobic interaction responsible for water solubility (Kovalcuks and Duma, 2014). Our results are consistent with Chung and Ferrier (1991) where they reported that 2:1 CM and other organic solvents cause denaturation to proteins that decreased functional properties up to 41%. In fact, the most significant decrease in water solubility occurred when 2:1 chloroform-methanol was used as the organic solvent in a lipid extraction method. The study also found that the different solvents denatured proteins at different rates which affected the solubility of the concentrated egg yolk proteins (Chung and Ferrier, 1991).

Gelling properties

Gelation of proteins is strongly dependent on changes in the protein structure and aggregation interactions; therefore, the ability to gel and the texture profiles of the gels made from the initial egg yolk powder and the concentrated egg yolk protein were compared. The gelling abilities and texture profiles were impacted by the type of organic solvent used. For example, CM and other organic solvents are known to cause denaturation to proteins and reduce their functional properties. Recall that the CM protein concentrates were the least soluble because the methanol altered the protein structure and increased surface hydrophobicity (Laca et al., 2015; Rickert and others, 2004); accordingly, when CM was used as the organic solvent, the

resulting protein concentrates did not form a solid gel. On the other hand, isopropanol and MTBE are less polar than methanol; therefore, they may not have caused the same extent of damage to the proteins and the ability to form gels remained intact.

Texture profile analysis

Texture profiles (hardness, springiness, gumminess, cohesiveness, and resilience) of the gels made from initial egg yolk and egg yolk protein concentrates from HI and MTBE were evaluated (Table 2). Gels were subjected to a first compression at 25% so they would not fracture during first compression. Hardness of a gel is measured by the peak force of the first compression to 25% of the original height. MTBE and HI gels were significantly harder ($p < 0.05$) than the gels prepared with the initial egg yolk powder likely due to the greater protein content in the concentrates. There may have been more protein networking without interference of lipids to form a harder gel. Cohesiveness of a gel is related to how well it holds together under two compression cycles; MTBE gels exhibited a significantly greater cohesiveness than HI and initial powder gels ($p < 0.05$). MTBE gels were also springier and gummier ($p < 0.05$) which is related back to the hardness of the gel. These results indicate that the MTBE gels would require more mastication energy to physically breakdown the product. Even though the protein concentrates obtained when HI was used as the organic solvent had a similar protein content as the MTBE, there was more lipid, which could explain the lower TPA values shown.

These results are similar to work done by Paraskevopoulou and Kiosseoglou (1997) where different solvents were used to remove lipid and cholesterol from egg yolk and the texture profiles of gels were compared. Similarly, the researchers employed the nonpolar petroleum ether and polar ethanol as a mixture and individually to extract the lipid. The TPA results were consistent with those in this current study where the defatted gels were harder and springier. The

authors attributed this to the higher protein content of the lipid extracted powders as well as lipoprotein constituents. The cohesiveness of the gels was decreased in the lipid extracted gels. The researchers used a compression to 50% which could have caused the gels to fracture. All TPA values increased with an increase in total solids within the gel (Paraskevopoulou and Kiosseoglou, 1997).

Water holding capacity

Water holding capacity (WHC) is a measurement of the water binding ability of the protein gels and impact many characteristics of a product including physical, chemical, and sensory attributes. WHC was measured by weight of the water lost during compression of the gels. Results are expressed as percent (%) water held in the gel (Figure 3). Overall, MTBE gels had the greatest water holding capacity ($p < 0.05$). Even though HI gels had greater values for TPA than gels made from the initial egg yolk, the WHC was significantly lower ($p < 0.05$). The protein gels from the HI lipid extracted powder did not bind and hold water as well as others. Hermansson (1994) indicated that microstructure of gels play a role in water holding capacity, and the finer texture of the MTBE powder could have created finer pore sizes in the gel to hold water due to more protein and water interactions from a finer dispersion. The water holding capacity of gels is also related to the texture of the gel meaning a higher strength gel will result in less water loss (Ayadi et al., 2008). MTBE was the strongest gel and resulted in the highest WHC.

Color

Color of a food product is an important characteristic for acceptance. Color measurements (L^* , a^* , b^*) of the gels were taken to measure differences between gels made with

protein concentrates and gels prepared with the initial egg yolk powder (Table 3). Even though the protein concentrates from extraction by 2:1 CM did not produce a gel, color measurements were still taken for comparison. The initial egg yolk powder was the lightest ($p < 0.05$) followed by gels prepared from HI and MTBE extracted protein, which had similar L^* values ($p > 0.05$). The initial egg yolk powder was also significantly more yellow than the other samples ($p < 0.05$). The color of egg yolk is generally attributed to the fat-soluble pigments such as carotenoid-like xanthophylls found within the yolk and is often important for quality of the product (Anton, 2007; Laca et al., 2010). The xanthophylls are obtained from corn or additives in food fed to poultry. β -carotene is a precursor to the lipid soluble vitamin A and is also related to the color of the yolk (Kovalcuks and Duma, 2014). Therefore, removing a portion of the lipid content also removed color attributes of the powder. Kovalcuks and Duma (2014) found that lipid extraction employing 2-propanol/hexane extracted more β -carotene than the ethanol/chloroform mixture. The CM gels in this study had less yellow color than the HI gels, but this could have been due to the CM gel having a redder color as well as being tested as a liquid rather than a gel like the HI sample. Warren and others (1988) investigated the effects of hexane, 2:1 HI, and 2:1 CM on the extraction of lipid from egg yolk solids. The authors found that HI and CM extracted roughly 70% of the β -carotene in the yolk (Warren et al., 1998). The MTBE defatted powder had the lowest percentage of lipid compared to HI, and therefore had a less intense yellow color than the gels with initial egg yolk powder.

Conclusion

The results of this study indicate that one-step organic solvent lipid extraction using hexane-isopropanol (HI) (3:2, v:v), chloroform-methanol (CM) (2:1, v:v), and methyl-tert-butyl ether (MTBE) has an effect on the functional properties of egg yolk protein concentrates.

Utilizing different solvent combinations ultimately reduce the lipid content of egg yolk powder while increasing the protein content. When CM and MTBE were used as the organic solvent, resulting protein concentrates had similar amounts of remaining lipid, but CM protein concentrates had the greatest protein content. However, the functional properties of CM protein concentrates were negatively affected by the solvents. MTBE extracted powders showed the best functional properties when compared to the initial egg yolk powder. The MTBE powder showed no change in water solubility, produced a stronger, harder gel, and had greater water holding capacity when compared to the other samples. HI extraction decreased functional properties of the egg yolk powder. This method shows that MTBE has potential to reduce lipid in egg yolk while keeping the functional properties egg yolk is known for. Future studies would look at ways to increase lipid extraction efficiency as well as looking at effects on other functional properties such as emulsifying capability and protein changes.

References

1. Alleoni, A. C. C. (2006). Albumen protein and functional properties of gelation and foaming. *Scientia Agricola*. 63(3):291-298.
2. Anton, M. (2007). Chapter 1: Composition and structure of hen egg yolk. Retrieved from: https://link.springer.com/content/pdf/10.1007%2F978-3-540-37885-3_1.pdf
3. Anton, M. (2013). Egg yolk: structures, functionalities, and processes. *Journal of Science Food Agriculture*. 93:2871-2880.
4. Association of Official Analytical Chemists (AOAC). (1995). Official methods of analysis, 16th ed.: Association of Official Analytical Chemists: Washington, D.C.
5. ASTM D 2974-87 Standard Test Methods for Moisture, Ash, and Organic Matter of Peat and Other Organic Soils. (1993). Retrieved from; <http://gsrpdf.lib.msu.edu/ticpdf.py?file=/1990s/1993/930331.pdf>
6. Ayadi, M.A., Khemakhem, M., Belgith, H., and Attia, H. (2008). Effect of moderate spray drying conditions on functionality of dried egg white and whole egg. *Journal of Food Science*. 76(6): E281-287.
7. Chung, S.L. and Ferrier, L.K. (1991). Partial lipid extraction of egg yolk powder: effect on emulsifying properties and soluble protein fraction. *Journal of Food Science*. 56(5):1255-1258.
8. Hermansson, A.M. (1994). Microstructure of protein gels related to functionality. In: *Protein structure-function relationship in foods*. London, UK.: Blackie Academic and Professional. 22-42.
9. Khmelnitsky, Y. L., Belova, A. B., Levashov, A. V., & Mozhaev, V. V. (1991). Relationship between surface hydrophilicity of a protein and its stability against denaturation by organic solvents. *FEBS letters* 284(2):267-269.
10. Kovalcuks, A. and Duma, M. (2014). Solvent extraction of egg oil from liquid egg yolk. 9th Baltic Conference on Food Science and Technology- Food for Consumer Well-Being: Foodbalt 2014:253-256.
11. Laca, A., Paredes, B., Rendueles, M., and Díaz, M. (2010). A method of egg yolk fractionation. Characterization of fractions. *Food Hydrocolloids*. 24(4):434-43.
12. Laca, A., Paredes, B., Rendueles, M., and Díaz, M. (2015). Egg yolk plasma: separation, characteristics, and future prospects. *LWT-Food Science and Technology*. 62(1):7-10
13. Lapanje, S. (1978). *Physicochemical aspects of protein denaturation*. John Wiley & Sons, New York, 142
14. Larsen, J.E., and Froning, G.W. (1981). Extraction and processing of various components from egg yolk. *Poultry Science*. 60(1): 160-167.
15. Marcet, I., Saez, S., Rendueles, M., Diaz, M. (2017). Edible films from residual delipidated egg yolk proteins. *Journal of Food Science and Technology*. 54(12):3969-3978.
16. Nakamura, R., Fukano, T., Taniguchi, M. (1982). Heat-induced gelation of hen's egg yolk low density lipoprotein (LDL) dispersion. *Journal of Food Science*. 47(5):1449-1453.
17. Paraskevopoulou, A. and Kiosseoglou, V. (1994). Cholesterol and other lipid extraction from egg yolk using organic solvents: effects on functional properties of yolk. *Journal of Food Science*. 59(4): 766-768.

18. Paraskevopoulou, A. and Kiosseoglou, V. (1997). Texture profile analysis of heat-formed gels and cakes prepared with low cholesterol egg yolk concentrates. *Journal of Food Science*. 62(1): 208-211.
19. Rickert, D. A., Johnson, L. A., Murphy, P. A. 2004. Functional properties of improved glycinin and beta-conglycinin fractions. *Journal of Food Science*. 69(4):C303–311.
20. Rose, A., Jaczynski, J., Matak, K. (2019a). Extraction of lipids from insect, krill, and egg yolk powders using a one-step organic solvent extraction process. Characterization of lipids and the protein co-products from various food sources using a one-step organic solvent extraction process. Master's thesis, West Virginia University.
21. Rose, A., Jaczynski, J., Mo Ku, K. Chiu Y., Matak, K. (2019b). Defatting insect powders to enhance protein quality and concentration. Characterization of lipids and the protein co-products from various food sources using a one-step organic solvent extraction process. Master's thesis, West Virginia University.
22. USDA National Nutrient Database for Standard Reference, Release 28. (2016) US Dept of Ag Research Services, Nutrient Data Laboratory. <https://ndb.nal.usda.gov/ndb/>
23. Valverde, D., Laca, A., Nel Estrada, L., Paredes, B., Rendueles, M., and Diaz, M. (2016). Egg yolk and egg yolk fractions as key ingredient for the development of a new type of gels. *International Journal of Gastronomy and Food Science*. 3:30-37.
24. Warren, Brown, and Davis. (1988). Solvent extraction of lipid components from egg yolk solids. *Journal of the American Oil Chemists' Society*. 65(7);1136-1139

Tables and Figures

Table 1. Proximate composition (percent protein, lipid, and ash are reported on dry matter basis) of initial egg yolk powder and protein concentrates separated by from lipid extraction using 2:1 chloroform-methanol (CM), 3:2 hexane-isopropanol (HI), and methyl-tert-butyl-ether (MTBE) as the organic solvent.

	Moisture	Protein	Lipid	Ash
Initial powder	3.15±0.12 ^C	36.09±0.16 ^C	49.47±0.93 ^A	6.31±0.04 ^C
CM	5.15±0.19 ^{AB}	66.19±0.55 ^A	25.67±0.40 ^C	9.17±0.41 ^{AB}
HI	4.80±0.27 ^B	57.27±0.63 ^B	27.70±0.64 ^B	9.38±0.12 ^A
MTBE	5.44±0.10 ^A	57.93±0.39 ^B	24.29±0.77 ^C	8.71±0.17 ^B

^{A,B,C} indicate significant differences ($p < 0.05$) in columns among the different egg yolk samples.

Table 2. Texture profile analysis of gels prepared with initial egg yolk powder and protein concentrates separated by lipid extraction using 2:1 chloroform-methanol (CM), 3:2 hexane-isopropanol (HI), and methyl-tert-butyl-ether (MTBE) as the organic solvent.

	Initial powder	HI	MTBE
Hardness (g)	183.65±10.34 ^b	642.70±81.95 ^a	654.74±75.41 ^a
Springiness (mm)	0.59±0.02 ^c	0.73±0.03 ^b	0.83±0.03 ^a
Cohesiveness (ratio)	0.57±0.01 ^c	0.60±0.01 ^b	0.66±0.01 ^a
Gumminess (g)	97.77±7.89 ^c	359.55±52.43 ^b	406.32±43.94 ^a
Resilience (ratio)	0.50±0.01 ^a	0.39±0.01 ^b	0.48±0.01 ^a

^{a,b,c} indicate significant differences ($p < 0.05$) in rows among the different egg yolk samples.

Table 3. Color measurements of gels prepared with initial egg yolk powder and protein concentrates separated by lipid extraction using 2:1 chloroform-methanol (CM), 3:2 hexane-isopropanol (HI), and methyl-tert-butyl-ether (MTBE) as the organic solvent.

	<i>L</i> *	<i>a</i> *	<i>b</i> *	Whiteness
Initial powder	90.98±0.75 ^A	-4.89±0.34 ^B	39.89±1.42 ^A	58.80±1.35 ^C
CM	77.25±1.40 ^C	0.39±0.25 ^A	29.45±0.83 ^C	62.77±1.15 ^B
HI	86.06±0.92 ^B	-4.54±0.30 ^B	32.66±1.24 ^B	64.19±1.00 ^{AB}
MTBE	84.62±1.33 ^B	-5.77±0.17 ^C	30.75±0.47 ^C	65.12±0.80 ^A

*L**, lightness (0–100); *a**, intensity of red color (–60 to +60); *b**, intensity of yellow color (–60 to +60)

^{A,B,C} indicate significant differences (*p*<0.05) in columns among the different egg yolk samples.

Figure 1. Recovery yields (%) of protein concentrates separated by lipid extraction using 2:1 chloroform-methanol (CM), 3:2 hexane-isopropanol (HI), and methyl-tert-butyl-ether (MTBE) as the organic solvent.

^{a,b,c} Different letters indicate significant differences ($p < 0.05$) among organic solvents.

Figure 2. Protein solubility (%) of initial egg yolk powder and protein concentrates separated by lipid extraction using 2:1 chloroform-methanol (CM), 3:2 hexane-isopropanol (HI), and methyl-tert-butyl-ether (MTBE) as the organic solvent.

^{a,b,c} Different letters indicate significant differences ($p < 0.05$) among organic solvents

Figure 3. Water holding capacity of gels prepared with initial egg yolk powder and protein concentrates separated by lipid extraction using 3:2 hexane-isopropanol (HI) and methyl-tert-butyl-ether (MTBE) as the organic solvent.

^{a,b,c} Different letters indicate significant differences ($p < 0.05$) among organic solvents

CHAPTER VI

APPENDIX

Foaming

Foaming properties were measured on initial egg yolk powder and protein concentrates in triplicates. Samples (5 grams) were reconstituted with distilled water to a dry matter content of 10% and allowed to sit for 3 min. The 50 mL solution was whipped using a handheld beater at speed level 5 for 2min. The whipped mixture was immediately poured into a 100 mL graduated cylinder to measure the volume of foam and liquid not incorporated. The volume of foam and liquid was measured at times 0, 5, 10, 15, 30, and 60 minutes. Foam capacity was calculated;

$$\text{Foam capacity} = \frac{\text{volume after whipping (mL)} - \text{initial liquid volume (mL)}}{\text{initial liquid volume (mL)}} \times 100$$

Results

Foaming properties

The egg yolk protein concentrate and initial powder were measured to determine if the protein unfolding or denaturation caused foaming properties. Foam expansion was calculated from observed results. After whipping the sample, the foam capacity for MTBE defatted egg yolk powder was calculated to be 60%. Compared to the initial egg yolk powder, which is not known to foam, it was an improvement of the functionality. This was most likely due to the MTBE unfolding proteins to allow them to produce foam while not being polar enough to damage protein interactions. The lipid in egg yolk is known to decrease foaming properties of egg whites in the food and culinary industries. Protein water solubility and degree of denaturation can play a significant role in foaming properties.