Egg yolk as a delivery system of omega-3 fatty acids

by

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ABSTRACT

Omega-3 fatty acids such as eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) have been related to multiple health benefits. One way to produce omega-3 enriched egg yolk is through hen's feed manipulation. However, there are many drawbacks to this approach: a high cost of production, wide variation in the amount of omega-3 fatty acids, fishy taste, and shortened shelf life. Thus, the purpose of this research was to investigate the feasibility of using a processing approach to produce egg yolk/fish oil emulsions with high content of EPA+DHA and to test its use as an ingredient in food products. The effects of fish oil concentration and esterification type, enzymatic modification of egg yolk, polysaccharide addition, and processing method on the emulsions were investigated. Emulsions were characterized by their viscosity, particle size and distribution, encapsulation efficiency, and stability during storage under different conditions. Consumer acceptance of EPA+DHA fortified ice cream and cake, using selected emulsions or non-encapsulated fish oil (control) as ingredients, was evaluated using a 9-point hedonic scale.

We found that the apparent viscosity of the emulsions increased after treatment of egg yolk with phospholipase A_1 (PLA_1) and with the addition of gum guar. The average particle size and distribution of egg yolk were affected by the concentration and esterification of fish oil, and processing conditions. In our first study, egg yolk was proved to be an efficient carrier of 1 and 5% (w/w) fish oil, showing 100% encapsulation efficiency, and lack of significant oxidation products formation after 4 weeks of storage at 4–6 °C. Results from our second and third studies showed that the encapsulation efficiency of emulsions containing up to 50% (w/w on egg dry matter basis) fish oil was significantly ($p < 0.05$) improved after secondary homogenization (200 MPa). Fish oil from emulsions showed lower formation of oxidation products during storage at

45 °C, compared to those found in non-encapsulated fish oil (up to 184.3 meq peroxide/kg oil and 9.7 μg propanal/g emulsion). None of the emulsions were toxic over 10 days of storage at 45 °C, whereas non-encapsulated fish oil decreased cell viability to 81%. In addition, the apparent permeability of EPA from emulsions in Caco-2:HT29 monolayers increased significantly (1.4-2.4 $x10^{-5}$) compared to that of non-encapsulated fish oil ($0.1x10^{-5}$). Furthermore, emulsions combining PLA_1 treatment and secondary homogenization were the most stable under high temperature conditions; whereas gum guar improved stability after freeze-thaw cycle. A significant preference of ice cream samples containing EPA+DHA from emulsions (6.5–7.1 flavor liking score) over control samples was found. Consumers noted fishy taste, unpleasant aftertaste, and rancid notes in control samples. Moreover, no significant differences were found in consumer preference of treatments tested on cake samples.

This thesis conclusively showed that secondary homogenization can be successfully used to produce egg yolk emulsions with high content of EPA+DHA. The stability of emulsions can be enhanced by enzymatic modification of egg yolk or with polysaccharides addition. Our findings increase the scope of applications for egg yolk particularly for use as a functional food ingredient.

PREFACE

This thesis is an original work by Selene Yadira Gonzalez Toledo. Chapter 2 of this thesis has been accepted for publication in the Journal of the American Oil Chemists' Society under the title "Encapsulation of long chain *n*-3 polyunsaturated fatty acids using egg yolk", DOI:10.1002/aocs.12299.

The sensory study in Chapter No. 6, which is a part of this thesis, received research ethics approval from the University of Alberta Research Ethics Board 2, Project Name "Consumer preference of omega-3 fortified food products", ID. Pro00065902_AME1, January 8th, 2019.

This research project was originated with the idea of Dr. Jianping Wu to fortify egg yolk with omega-3 fatty acids using a processing approach to overcome the hen's feed manipulation issues. Selene Gonzalez gathered the theoretical information and developed a strategic plan to achieve the goal as reported in Chapters 2 and 3. After obtaining successful and promising results in the first stage of the research, Selene Gonzalez developed the experimental designs used to move forward with the research as described in Chapters 4 and 5, under the supervision of Dr. Jianping Wu. Chapter 6 of this thesis, specifically the consumer acceptance study, was developed with the advice of Dr. Wendy Wismer.

All the experiments and statistical analyses of this research were conducted by Selene Gonzalez, with exception of quantification of fatty acids methyl and ethyl esters by gas chromatography, and propanal by head-space chromatography, which were conducted at the Chromatography laboratory from the Department of Agricultural, Food and Nutritional Science at the University of Alberta, in charge of chromatography technologist Lisa Nicolai. The experimental processes and the writing of this thesis were at all times reviewed and corrected by Dr. Jianping Wu.

DEDICATION

To my sister Mirna,
my greatest example of perseverance

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1%EE = Emulsion containing 1% fish oil ethyl esters

1%TG = Emulsion containing 1% fish oil triglycerides

5%EE = Emulsion containing 5% fish oil ethyl esters

5%TG = Emulsion containing 5% fish oil triglycerides

A22EH = Egg yolk/gum arabic emulsions containing 22% fish oil ethyl esters, formed by high-pressure homogenization

A22TH = Egg yolk/gum arabic emulsions containing 22% fish oil triglycerides, formed by high-pressure homogenization

A22ES = Egg yolk/gum arabic emulsions containing 22% fish oil ethyl esters, formed by simple homogenization

A22TS = Egg yolk/gum arabic emulsions containing 22% fish oil triglycerides, formed by simple homogenization

A44EH = Egg yolk/gum arabic emulsions containing 44% fish oil ethyl esters, formed by high-pressure homogenization

A44TH = Egg yolk/gum arabic emulsions containing 44% fish oil triglycerides, formed by high-pressure homogenization

A44ES = Egg yolk/gum arabic emulsions containing 44% fish oil ethyl esters, formed by simple homogenization

A44TS = Egg yolk/gum arabic emulsions containing 44% fish oil triglycerides, formed by simple homogenization

ALA = Alpha-linolenic acid

CFR = Code of Federal Regulations

Control-AH = Egg yolk/gum arabic emulsions without fish oil, formed by high-pressure homogenization

Control-AS = Egg yolk/gum arabic emulsions without fish oil, formed by simple homogenization

Control-GH = Egg yolk/gum guar emulsions without fish oil, formed by high-pressure homogenization

Control-GS = Egg yolk/gum guar emulsions without fish oil, formed by simple homogenization

DHA = Docosahexaenoic acid

DLS = Dinamic light scattering

EE = Ethyl esters

EEf = Encapsulation efficiency

EPA = Eicosapentaenoic acid

EY = Egg yolk

FAO = Food and Agriculture Organization of the United Nations

FO = Non-encapsulated fish oil triglycerides

G22EH = Egg yolk/gum guar emulsions containing 22% fish oil ethyl esters, formed by high-pressure homogenization

G22TH = Egg yolk/gum guar emulsions containing 22% fish oil triglycerides, formed by high-pressure homogenization

G22ES = Egg yolk/gum guar emulsions containing 22% fish oil ethyl esters, formed by simple homogenization

G22TS = Egg yolk/gum guar emulsions containing 22% fish oil triglycerides, formed by simple homogenization

G44EH = Egg yolk/gum guar emulsions containing 44% fish oil ethyl esters, formed by high-pressure homogenization

G44TH = Egg yolk/gum guar emulsions containing 44% fish oil triglycerides, formed by high-pressure homogenization

G44ES = Egg yolk/gum guar emulsions containing 44% fish oil ethyl esters, formed by simple homogenization

G44TS = Egg yolk/gum guar emulsions containing 44% fish oil triglycerides, formed by simple homogenization

GRAS = Generally Recognized As Safe

HDL = High-density lipoproteins

LDL = Low-density lipoproteins

***n*-3** = Omega-3

***n*-3 LC-PUFAs** = Omega-3 long-chain omega-3 polyunsaturated fatty acids

PLA_1 = Phospholipase A_1

PLA_2 = Phospholipase A_2

PLD = Phospholipase D

POV = Peroxide value

P17H = Emulsion containing 17% fish oil using PLA_1-treated egg yolk as carrier formed by high-pressure homogenization

P29H = Emulsion containing 29% fish oil using PLA_1-treated egg yolk as carrier formed by high-pressure homogenization

P38H = Emulsion containing 38% fish oil using PLA_1-treated egg yolk as carrier formed by high-pressure homogenization

P44H = Emulsion containing 44% fish oil using PLA_1-treated egg yolk as carrier formed by high-pressure homogenization

P50H = Emulsion containing 50% fish oil using PLA_1-treated egg yolk as carrier formed by high-pressure homogenization

R17H = Emulsion containing 17% fish oil using raw egg yolk as carrier formed by high-pressure homogenization

R29H = Emulsion containing 29% fish oil using raw egg yolk as carrier formed by high-pressure homogenization

R38H = Emulsion containing 38% fish oil using raw egg yolk as carrier formed by high-pressure homogenization

R44H = Emulsion containing 44% fish oil using raw egg yolk as carrier formed by high-pressure homogenization

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P50S = Emulsion containing 50% fish oil using PLA_1-treated egg yolk as carrier formed by simple homogenization

R17S = Emulsion containing 17% fish oil using raw egg yolk as carrier formed by simple homogenization

R29S = Emulsion containing 29% fish oil using raw egg yolk as carrier formed by simple homogenization

R38S = Emulsion containing 38% fish oil using raw egg yolk as carrier formed by simple homogenization

R44S = Emulsion containing 44% fish oil using raw egg yolk as carrier formed by simple homogenization

R50S = Emulsion containing 50% fish oil using raw egg yolk as carrier formed by simple homogenization

PSD = Particle size and distribution

TG = Triglycerides

CHAPTER 1. INTRODUCTION

Dietary habits are a key contributor to the high prevalence of metabolic and cardiovascular diseases. One approach to mitigate the epidemics of these diseases is to incorporate bioactive compounds as part of a regular diet. Long-chain omega-3 polyunsaturated fatty acids (*n*-3 LC-PUFAs), mainly in the form of eicosapentaenoic (EPA) and docosahexaenoic (DHA) fatty acids, are leading the way among bioactive compounds with potential benefits in the prevention and treatment of metabolic and cardiovascular diseases.

EPA and DHA have been associated with the prevention and control of cancer, hypertriglyceridemia, hypertension, arthritis and other autoimmune and inflammatory disorders (Jordan and Stein, 2003; Micaleff et al., 2009; Howe and Buckley, 2014; Hedengran et al., 2015). In addition, DHA is essential for the development of the brain and the nervous system of fetuses and children. Therefore, multiple health authorities have recommended the consumption of these nutrients. For example, the Food and Agriculture Organization of the United Nations recommends a minimum daily intake of 150 mg of EPA+DHA for the optimal brain development of children and 250 mg for healthy adults (FAO, 2008). In addition, the American Heart Association recommends a weekly consumption of 7 ounces of cooked fish, and the Food and Drug Administration has set a GRAS (Generally Regarded As Safe) level of 3000 mg/day of EPA+DHA (CFR, 1997).

Nonetheless, despite the importance of consuming *n*-3 LC-PUFAs, their levels in the average North American diet remain low. One of the main concerns related to the consumption of certain types of fish is the risk of contamination with heavy metals from industrial pollutants, such as mercury. Therefore, food scientists are focusing on developing food and food products with increased levels of these essential nutrients from sources that are safe for human consumption.

However, there are several issues related to the enrichment of food products with omega-3 polyunsaturated fatty acids; lipid oxidation being the main concern. Lipid oxidation causes a negative impact on the nutritional content and overall quality of food. For this reason, the use of a delivery system that preserves the lipids until metabolization and absorption by the human body is of vital importance.

Throughout this research project we collected and analyzed the various studies that helped us investigate and explain egg yolk's potential as a delivery system of *n*-3 LC-PUFAs. Egg yolk was selected not only for its widely known nutritional and functional properties but because eggs are one of the most common foods consumed around the world. Consequently, using egg yolk as carrier of *n*-3 LC-PUFAs would allow more people to increase their consumption of these essential fatty acids without any major changes in their dietary habits. In addition, this also represents an opportunity to develop value-added functional egg products that can help the Canadian egg industry grow. The egg industry constitutes an important part of the agricultural economy. For instance, in 2017, egg farms in Canada contributed 1.7% cash receipts to the total farming operation. Approximately 73% of the total production accounted for table eggs, while the remaining 27% was further processed. Over the past decade, a steady increase of egg consumption in Canada and North America has been observed. In Canada, the annual hen's egg per capita consumption reached 20.2 dozen in 2017, representing an increase of 1.5 percent from 2016 (Agriculture and Agri-Food Canada, 2018). One of the main reasons for this trend is the change in consumer attitude towards eggs: people are now more aware that cholesterol from their diet has little impact on their plasma cholesterol levels (Larsson et al., 2015; Virtanen et al., 2016). Thus, eggs are now included in protein-rich diets. An important advantage eggs have over other low-cost protein sources is their protein quality, which provides all nine essential amino acids for humans. Additionally, eggs also provide a well-balanced mix of nutrients such as vitamins, minerals and essential fatty acids (Huopalahti et al., 2007). Aside from their nutritional advantages, eggs are also an important commodity in the food industry because of their functional properties.

In this study, we hypothesize that egg yolk as a natural emulsifier can be used to encapsulate and protect *n*-3 LC-PUFAs from oxidation. In order to prove our hypothesis, the objective of this research was to investigate the feasibility of using a processing approach to produce egg yolk with high content of EPA+DHA, and to assess its impact on sensory characteristics when used as an ingredient in food products. Figure 2.1 illustrates the schematic process followed for the development of this research. We began this research with the raw material selection, followed by the use of primary homogenization to develop an oxidative stable egg yolk product that provides at least 100% of the recommended daily intake of EPA+DHA in one serving (30 g). In spite of the positive and promising stability results from our first study, the

consumer acceptance of the developed egg yolk product was the main drawback. Therefore, we moved forward to a second and third study to assess the effect of treatment with phospholipase A_1, gum guar or gum arabic addition, and primary and/or secondary homogenization on the stability of egg yolk/fish oil emulsions containing up to 50% of fish oil. We hypothesized that enzymatic modification with PLA_1, and the inclusion of neutral or anionic polysaccharides can improve the encapsulation efficiency and stability of the emulsions at higher storage temperatures. After successfully developing stable emulsions, we selected three treatments to test their potential use as food ingredients. Thus, in our fourth and final study, we hypothesized that EPA+DHA encapsulated using egg yolk-based emulsions as carriers can be more resistant to degradation during processing conditions than those from non-encapsulated fish oil. Therefore, our final objective was to test the stability of the developed egg yolk-based emulsions in two food systems: ice cream and cake. We fortified ice cream and cake with EPA+DHA from emulsions and non-encapsulated fish oil (control) and assessed the consumer acceptance. During this final study, the stability of EPA+DHA during processing and storage was also evaluated.

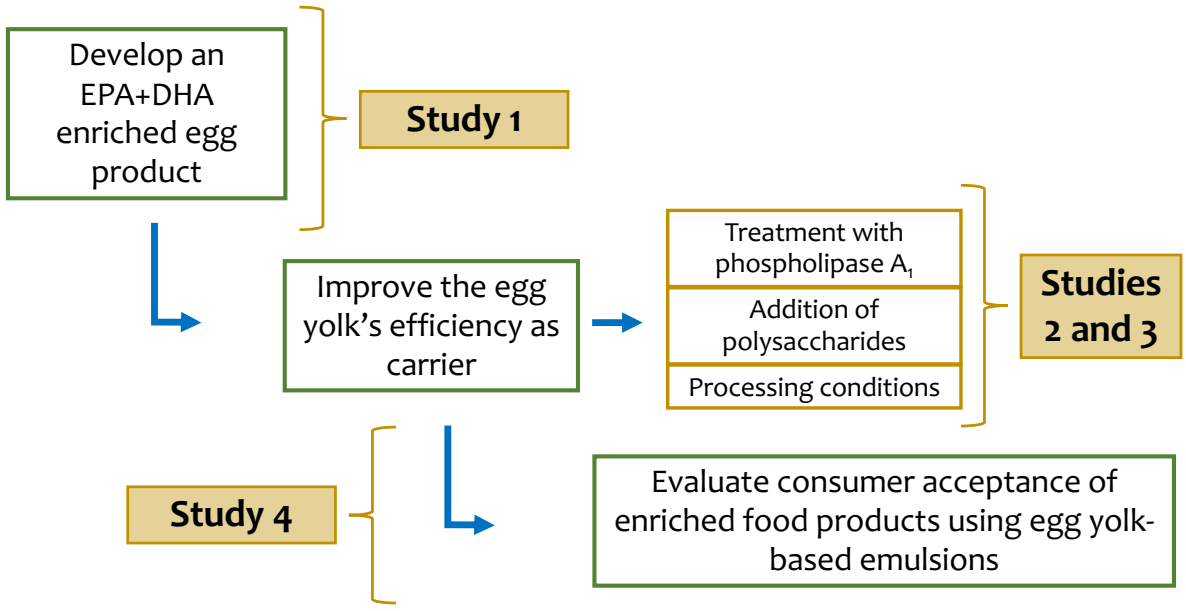

Figure 1.1 Schematic representation of the research development to use egg yolk-based emulsions as delivery systems of omega-3 fatty acids.

CHAPTER 2. LITERATURE REVIEW

2.1 The hen's egg yolk

2.1.1 Structure and composition

Hen's egg (hereby referred to as "egg") structure is composed of egg yolk enclosed by a transparent membrane called vitelline membrane, surrounded by the albumen, and balanced by the chalaza. The major chemical components of the yolk are α- and β-lipovitellins, phosvitin, low-density lipoproteins, livetins, inorganic ions, and some enzymes (Bellairs and Osmond, 2014).

The egg yolk is constituted of two main fractions: an insoluble micro-fraction, also called granules, and a water-soluble nano-fraction or plasma (Figure 2.2). At low ionic strength the high negative charge of phosvitin leads to aggregation with high-density lipoproteins (HDL) to form insoluble complexes, or granules, linked by phosphocalcic bridges. These granules consist of about 70% HDL, 16% phosvitin, and 12% low-density lipoproteins; they have been reported to contain less phospholipids, cholesterol and triglycerides than plasma (Jin et al., 2013). The plasma fraction accounts for 90% of total lipids in egg yolk and is composed of 85% low-density lipoproteins (LDL) and 15% livetins on dry matter basis (Burley and Cook, 1961; Anton and Gandemer, 1997).

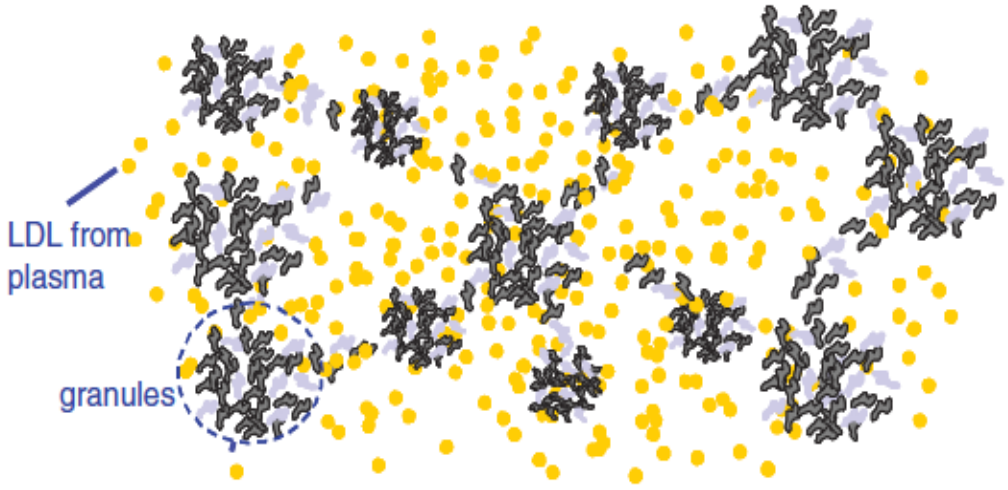

Figure 2.1 Micro and nano-fractions of hen's egg yolk (from Anton et al., 2013).

2.1.2 Functional properties

Egg yolk is widely known to be an excellent emulsifier. Its complex protein-based emulsifying system together with other functional properties such as its coloring, antioxidant, coagulant, and gelling capacity makes the egg yolk a highly-valued commodity for the food industry.

The flexible molecular structure and great surface hydrophobicity of egg yolk lipoproteins confer their high adsorption capacity at the oil-in-water interface (Mine, 1998). This characteristic is directly related to the emulsifying properties of egg yolk. However, this interface is influenced not only by the type of proteins, but also by the degree of denaturation, and the process used to form the emulsion (Anton and Gandemer, 1999; Ayadi et al., 2008). For instance Ayadi et al. (2008) showed that, emulsions formed using spray-dried eggs have lower stability, measured by the weight loss (%) after centrifugation at 10000 x *g* for 30 min, when processed at 125 °C and a flux of 0.2 l/h in comparison with processing at a flux of 0.3 l/h. Nevertheless, both treatments showed a higher emulsion stability compared to fresh egg. This behavior was attributed to the thermal treatment which caused unfolding of LDL apoproteins resulting in an increased hydrophobicity and molecular flexibility.

Heat induced egg yolk gelation is a multistage process, including initial protein denaturation, aggregation of unfolded proteins by hydrophobic interactions and random association of aggregates stabilized by covalent disulphide linkages involving granule proteins (Le Denmat et al., 1999). When egg yolk is exposed to temperatures of 72-76 °C, livetines, the most heat sensitive egg yolk proteins, unfold and interact with the partially denatured LDL, causing the first viscosity maximum. The second viscosity maximum is observed at temperatures above 85 °C when granule proteins are denatured and aggregated. Changes caused by heat treatment will affect the functional properties of egg yolk. Therefore, it is very important to know and consider these parameters when designing and establishing conditions to process egg yolk for use as a functional ingredient.

The coloring property of egg yolk is mainly attributed to lipophilic components such as lutein, zeaxanthin, β-cryptoxanthin, and β-carotene, mostly present in the plasma fraction (Li-Chan and Kim, 2008).

2.2 Bioactive compounds in eggs and their role in human health

A bioactive compound can be defined as those components that provide specific health benefits to humans (McClements et al., 2007). The regular consumption of these bioactive compounds is considered vital for health maintenance (Gaine et al., 2013). Some essential nutrients, such as *n*-3 LC-PUFAs, can be considered bioactive compounds as they are capable of modulating metabolic processes that result in the improvement of human health. Some bioactive compounds naturally occur in foods. The importance of these components has opened the path to further research into the production of bioactive components from their natural source, for example: inducing enzymatic digestion of proteins, or the inclusion of these compounds to increase their levels in foods and food products. For the purpose of this review, we will focus on the two main lipophilic bioactive compounds from eggs: *n*-3 polyunsaturated fatty acids and carotenoids.

2.2.1 Omega-3 polyunsaturated fatty acids

This group of bioactive compounds include medium or long-chain highly unsaturated fatty acids (Figure 2.3), all considered essential, since the human body cannot synthetize them. However, due to their potential benefits to human health, *n*-3 LC-PUFAs, mainly in the form of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are currently leading the list of nutraceutical components in products for human consumption. Among other benefits, EPA and DHA have shown anticarcinogenic potential (Jordan and Stein, 2003), help reduce triacylglycerols in plasma (Hedengran et al., 2015), improve carbohydrate metabolism (Ahmed et al., 2014), decrease the risk of cardiovascular disease and other diseases affected by immune response disorders, such as type 2 diabetes, inflammatory bowel diseases, and rheumatoid arthritis (McClements et al., 2007).

The omega-3 (*n*-3) refers to the location of the first double bond: the third carbon from the methyl end of the molecule. Alpha-linolenic acid (ALA), is an 18-carbon fatty acid with three *cis* double bonds, a medium-chain fatty acid that belongs to the *n*-3 PUFAS category. ALA can be obtained in small proportions from marine sources such as fish, algae and krill; higher proportions can be found in terrestrial sources such as flaxseed, walnuts, canola, chia, and soybeans (Arantes et al., 2009; Coorey et al., 2015). However, in order to display the health benefits in humans, ALA needs to be converted into its bioactive forms, EPA and/or DHA, belonging to the *n*-3 LC-PUFAs group.

Unlike ALA, the main sources of EPA and DHA are marine products. Fatty fish such as salmon, trout, herring, and mackerel are the major dietary sources of EPA and DHA. Nevertheless, other seafood, such as shrimp and mussels, also contain significant amounts of these fatty acids; and more recently, metabolic engineering of microorganisms has also shown potential to produce these fatty acids (Xue et al., 2013). These *n*-3 PUFAS could be also found among the lipids naturally present in egg yolk. However, eggs are not a significant source of EPA and DHA since their presence is highly dependent on the hen's diet (Coorey et al., 2015) and its content per portion is below the recommended daily consumption. Therefore, it is important to develop technologies to increase the content of these compounds in eggs and other food products. This topic is discussed in detail in section 2.5.

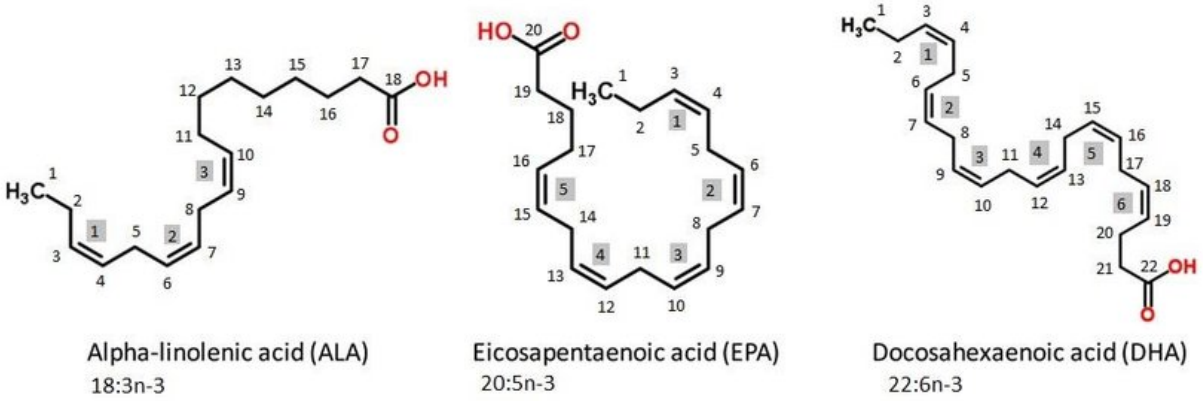

Figure 2.2 Chemical structure of the omega-3 fatty acids main components: ALA, EPA, and DHA (from Roke, 2017).

EPA is a 20-carbon fatty acid with 5 double bonds and its benefits are mostly associated with improved cardiovascular and immunological health (Ryan et al., 2010). The longer chain *n*3-PUFA, DHA is a 22-carbon fatty acid with 6 double bonds that has been directly related to brain and nervous system development and health. DHA represents about 15% of the fatty acids in the brain and is contained in the phospholipids that form the membrane structure of neurons. It has the ability to be retained in the plasma membrane of erythrocytes for the entire life of the cells (Brown et al., 1991). Among the *n*-3 fatty acids, DHA has been strongly associated with the reduction of body fat (Micaleff et al., 2009). For instance, the contents of DHA in fatty acids from erythrocytes was found to be inversely associated with body mass index, waist

circumference and body fat percentage in a sample of 476 adults. It was also observed that female participants showed higher erythrocyte DHA than males, suggesting a gender influence on the effect of *n*-3 LC-PUFAs on adiposity (Howe and Buckley, 2014). EPA and DHA have also been shown to improve the transepithelial transport of calcium in Caco-2 cells monolayers (Gilman and Cashman, 2007), indicating their potential to improve intestinal calcium absorption.

2.2.2 Carotenoids

Carotenoids are natural pigments present in a wide variety of fruits, vegetables, flowers, leaves, and also in some animal-derived products such as egg yolk. These pigments range from yellow to red. Humans and animals are unable to synthetize carotenoids, therefore, these need to be obtained from their diet.

Carotenoids play an important role in improving human health. Lutein, a component of the carotenoid's family, has been shown to have a positive effect in the prevention and reduction of cataracts and macular degeneration of the eye (Dawczynski et al., 2013). In addition, lutein has shown potential to protect against cardiovascular diseases, and some types of cancer, such as breast, lung, and skin cancer (Ribaya-Mercado and Blumberg, 2004). The relative bioavailability of lutein has been reported to be 67% (van HetHof et al., 2000).

2.3 Issues related with the use of lipophilic bioactive compounds

2.3.1 Bioavailability

An important parameter to consider when evaluating the effects of EPA and DHA in human health is their bioavailability. Usually, only a fraction of the total amount ingested of these compounds is absorbed by the human body (Ghasemifard et al., 2015).

Bioavailability can be defined as the rate of digestion, absorption, excretion, and deposition in tissues of the bioactive compound. This parameter could depend on three main factors: esterification type (free fatty acid, triglycerides, or ethyl esters), total dosage and frequency of consumption, and the matrix that acts as delivery system.

Although the role of esterification type has not been well defined, some researchers have found that results could depend on the subject tested as well as the concentration and carrier of the fatty acids. Hedengran et al. (2015) reported no significant difference in the effect of EPA+DHA as acylglycerol or ethyl esters in the reduction of non-fasting plasma triacylglycerol

in 120 subjects with hypertriglyceridemia. In addition, they found a negative correlation between triacylglycerol and EPA+DHA levels in plasma.

Furthermore, it has been reported that the dosage and frequency of consumption have a significant impact on the bioavailability of EPA, DPA and DHA from fish oil. Ghasemifard et al. (2015) reported a lower bioavailability (deposition) of EPA, DPA, and DHA in male rats after consuming a constant daily dose in comparison to a weekly consumption accounting for a 7 days dosage. An 84% β-oxidation was observed for the total dietary EPA+DPA+DHA from the weekly feeding treatment, resulting in a deposition of 25%. On the other hand, rats fed with a small daily dose of *n*-3 LC-PUFAs showed a deposition of only 15%. Thus, the main fate of the *n*-3 LC-PUFAs was shown to be catabolism (β-oxidation).

In order to present health benefits, *n*-3 LC-PUFAs need to be deposited in body tissues. The percentage of bioactive compounds that can reach this final fate could be improved with the use of a delivery system that can protect the compound during its extraction, incorporation in the food matrix, processing, and through the gastrointestinal tract. Thus, a wide variety of ingredients that could serve this purpose have been studied.

2.3.2 Oxidation

The chemical structure of lipophilic bioactive compounds makes them prone to oxidation. A clear example is the multiple double bonds in the *n*-3 LC-PUFAs, which make them especially susceptible to react with oxygen. For instance, it has been shown that the higher number of double bonds in DHA give it a higher rate of autoxidation compared to EPA (Lee et al., 2003). Oxidation decreases the shelf life, consumer acceptability, functionality, and nutritional value of the food product (Arab-Tehrany et al., 2012). Primary oxidation products, such as hydroperoxides, can decompose to secondary products such as aldehydes, ketones, acids, and alcohol, which causes characteristic off flavors and odors (Jacobsen and Timm, 2001; Lee et al., 2003). Aldehydes such as (E,E)-2,4-heptadienal and (E,Z)-2,6-nonadienal, have been detected in fish oil and have been associated with off flavors described as oxidized, painty, and green (Karahadian and Lindsay, 1989). Oxidation of oils are also reflected in color changes, an oxidized lipid develops a darker yellow or brown color (Duan et al., 2011; Salminen et al., 2014).

Moreover, oxidation of oils also carries health concerns. Some of the secondary oxidation products of unsaturated fatty acids, such as malondialdehyde and 4-hydroxynonenal, have been linked with aging, mutagenesis, and carcinogenesis (Nair et al., 1986, Nicolson and Ash, 2014).

2.3.3 Solubility

Various bioactive components are highly lipophilic, which presents technical difficulties when looking to develop water-based nutraceutical products. Hence, these components first need to be incorporated into a delivery system that can act as a carrier (McClements et al., 2009; Yang and McClements, 2013). Compounds that present amphiphilic properties are desirable to entrap lipophilic compounds to increase their solubility in aqueous systems. This is an important property of encapsulating materials because it will define their scope of applications in food processing. Egg yolk lipoproteins show amphiphilic properties that could be used to entrap lipophilic bioactive compounds thus making them soluble in aqueous systems.

2.4 Food delivery systems

During the development of delivery systems, several factors such as type of wall material, processing technology and conditions, and the oil-to-wall material ratio should be considered. These factors are of significant importance since the outcomes of the research will be based on them. The ideal carrier of bioactive compounds for food applications should present the following characteristics: food-grade, cost-effective production, chemical compatibility with different food matrixes, high payload and encapsulation efficiency, high emulsion stability during processing and storage, small particle size, low oxygen permeability, increased bioavailability of the components after ingestion, and most importantly, it should not adversely alter the quality characteristics of the final food product (Gulotta et al., 2014; Ramakrishnan et al., 2013; Yang and McClements, 2013).

The main purpose of developing a food-grade delivery system is to develop nutraceutical food products that will provide extra health benefits to the final consumer. The characteristics mentioned in section 2.2.1 make *n*-3 LC-PUFAs especially important bioactive compounds, therefore, several studies have been conducted to create a high-efficiency delivery system of these nutrients (Kassis et al., 2010; Duan et al., 2011; Matalanis et al., 2011; Gulotta et al., 2014).

2.4.1 Encapsulating materials commonly used as delivery system of bioactive components

In an effort to produce a highly efficient delivery system, different wall materials have been tested, single or combined. Among the most reported ingredients to form nano and micro-capsules, for delivery of bioactive compounds, are proteins such as sodium caseinate (Chung et al., 2010; Alvarez et al., 2014), hydrolyzed whey protein and whey protein isolate (Ramakrishnan et al., 2013), β-lactoglobulin (Frede et al., 2014); lipids such as liposomes (Hadian et al., 2014) and tryglicerides (Salminen et al., 2014); and polysaccharides such as chitosan (Duan et al., 2011; Jimenez-Martin et al., 2015), xanthan gum and pectin (Xu et al., 2017), alginate and locust bean gum (Quin et al., 2016).

Sodium caseinate in combination with high-amylose resistant starch has been used to encapsulate fish oil. Chung et al. (2010) reported that the wall material had a significant impact on the oxidative stability of fish oil. The combination of sodium caseinate with pre-heated and microfluidized resistant starch resulted in the highest encapsulation efficiency obtained for all treatments tested; however, contrary to what was expected, the same treatment had the lowest oxidative stability. This behavior was attributed to the low efficiency of the wall material to act as a barrier to oxygen.

Alvarez et al. (2014) studied the encapsulation efficiency and oxidative stability of fish oil microcapsules using a combination of threalose as cryoprotectant and sodium caseinate as emulsifier. They found that the most stable microcapsules were obtained when combining 20% threalose with 5% sodium caseinate. These microcapsules presented a constant particle size and distribution, before and after freeze-drying, and in the reconstituted powder. This combination also allowed the maximum encapsulation efficiency (79.4±0.9) among all treatments tested. The relatively small encapsulation efficiency led to high peroxide values (95.4±7.2 meq/kg). Contrary to what was expected, the higher encapsulation efficiency also presented the higher oxidation degree. This behavior was later found to be due to the extraction process of the core material which caused oxidation. Therefore, the higher the percentage of core material, the higher degree of oxidation due to the extraction process. One important consideration from this work that was not studied was the impact of adding threalose in the sensory quality of the product.

The unique physicochemical properties of phospholipids allow them to form bilayer membranes arranged in circular hollow structures, called liposomes. Hadian et al. (2014) used 1,2-dipalmitoyl-sn-glycero-3-phosphocholine liposomes to encapsulate pure EPA and DHA; they found that probe sonication reduces the particle size of the loaded liposomes and increases the encapsulation efficiency. In addition, they found that the liposome efficiency to encapsulate DHA was higher (56.9±5.2%) than EPA (38.6±1.8%). This observation was attributed to the DHA's natural ability to be incorporated in liposome membranes. Saturated triglycerides, such as tristearin, have also been used to carry fish oil with up to 30% of n-3 LC-PUFAs content (Salminen et al., 2014).

Anionic, cationic, and neutral polysaccharides have been recently used to encapsulate lipophilic and hydrophilic compounds. They have been tested in single form or in combination with proteins to provide an extra barrier to protect the core material and increase the stability of the emulsions. Chitosan, a linear polysaccharide derived from chitin, has been proven to be an effective encapsulating material of β-carotene and brilliant blue (Akbari and Wu, 2016). In another study performed by Duan et al. (2011), chitosan – whey protein isolates films were used to encapsulate EPA from fish oil. The treatments tested achieved around 50% of encapsulation efficiency; the high concentration of fish oil that remained free in the films was rapidly oxidized, as shown by the malonaldehyde value (330.5 mg MA eq / kg) in films containing 28.1% fish oil. Although these values do not seem promising in the development of a delivery system, the inclusion of 0.5% oregano oil reduced oxidation during processing up to 50%; moreover, films were stable after 30 days of storage showing no changes in the original encapsulation efficiency and oxidation values. Chitosan has also been used as emulsifier in a combination with lecithin to form multilayered fish oil emulsions with maltodextrin as wall material (Jimenez-Martin et al., 2015). Alginate and chitosan were found capable of inhibiting the hydrolysis of corn oil in an in vitro small intestine model (Qin et al., 2016). It has been shown that some proteins can form multilayer emulsions with polysaccharides leading to an increased stability by preventing aggregation (Xu et al., 2017). These findings could be applied to the formation of multilayered egg yolk emulsions for the encapsulation of lipophilic bioactive compounds.

2.4.2 Aids in the formulation of delivery systems

The common problem in most of the studies reported in section 1.5.1 is the low stability of the emulsions, as well as the lack of efficiency of the wall materials to act as oxygen barriers, hence the need to use surfactants and antioxidants to increase the shelf-life of the products.

2.4.2.1 Antioxidants

Antioxidants can prevent or retard lipid oxidation by free-radical scavenging activity, transition-metal-chelating activity, and/or singlet-oxygen-quenching capacity.

Among the antioxidants commonly used as aids for delivery systems there are chemical and natural-source antioxidants such as rosemary and oregano oils (Duan et al., 2011). Hydrophilic compounds with antioxidant effect, such as ascorbic acid, have been used to protect *n*-3 PUFAs against oxidation. The antioxidant effect of ascorbic acid is mainly by oxygen and hydrophilic free radicals scavenging (Kläui and Pongracz, 1981). Nevertheless, ascorbic acid also presents prooxidant effect due to its ability to reduce Fe^{+3} to Fe^{+2}, which can further catalyze the breakdown of existing lipid hydroperoxides. This behavior was studied by Jacobsen and Timm (2001). They observed that the addition of ascorbic acid in fish oil-enriched mayonnaise promoted the iron release from the oil-in-water into the aqueous phase of mayonnaise at different pH values when stored at 20 °C for up to 4 weeks. This effect caused flavor deterioration in the stored samples, as reflected by the positive correlation of *n*-3 LC-PUFAs oxidation products, such as pentanal, hexanal, heptanal, and 2-E-heptenal.

2.4.2.2 Emulsifiers for food systems

Generally, when developing an oil-in-water emulsion to act as carrier for lipophilic compounds, the use of surfactants is necessary to increase stability of the emulsions by preventing aggregation. In these situations, the surfactant selected plays a crucial role in the bioavailability and absorption of the bioactive compound. The most common surfactant is lecithin (from soy or eggs); however, bile salts, saponins (Yang and McClements, 2013; Salminen et al., 2014; Bai et al., 2016), tween 20 (Ramakrishnan et al., 2013; Frede et al., 2014), and Tween® 80 have also been used. The disadvantage of using surfactants such as Tween® 20 and 80, is that their use in food products is regulated. For instance, the World Health Organization has suggested daily intake limits of 0 – 25mg per kg of body weight. In addition, the FDA has established a

maximum limit of 0.1% of tween 80, or polysorbate 80, in most food products. In addition, Tween 20 has been shown to form bigger droplet size when emulsifying lutein, in comparison with other emulsifiers, such as β-lactoglobulin (0.61 and 0.32, respectively); it also showed less resistance to creaming during centrifugation at 1250 x *g* for 1 h at 20 °C (Frede et al., 2014).

Although antioxidants and surfactants have been shown to increase the stability of oil-in-water emulsion, an important parameter that is commonly overlooked is their impact in the sensory characteristics of the product. For example, natural antioxidants such as rosemary and oregano oil are known for being highly aromatic, characteristic that is transferred and reflected in the final product. In addition, surfactants as Tween® 20 and 80 present high bitterness, an undesirable characteristic in most food products.

2.4.3 Processing technologies applied to the development of delivery systems

The technology applied during the formation of delivery systems should be carefully chosen since it will impact the final characteristics of the emulsions. Microencapsulation is a technique where the core material, usually a solid or liquid molecule, is surrounded by the wall material which is meant to protect it against its environment. Usually, microencapsulation and nanoencapsulation differ only by the particle size. Generally, capsules with a size <100 nm are considered in the nano range; whereas microcapsules can vary from 100 nm to 5 μm. However, the technologies involved in the production of delivery systems will have a significant impact on the final size and properties of the capsules. One of the most common methods to produce microcapsules is spray drying. Although this method is one of the cheapest methods employed, it comes with the disadvantage of using high temperatures and allowing oxygen access throughout the process, most of the time causing degradation of heat-sensitive compounds.

The spray drying counterpart, freeze drying, offers more protection for sensitive compounds due to its low temperatures and oxygen-free environment. However, it is high-cost and time consuming. Microcapsules resulting from freeze drying have a narrow spectrum of applications in food industry, such as low-moisture and modified atmosphere packaging. It has been shown that adding a cryoprotectant prevents particle aggregation during freeze drying; however, adding 30% threalose had a negative impact in the particle distribution after reconstitution of threalose-sodium caseinate fish oil powders, by going from monomodal to bimodal particle distribution (Alvarez et al., 2014).

Edible films have also been studied for its potential to deliver bioactive compounds. Although in this method there is no capsules formation and the scope of applications is more limited, some studies have shown success when using for delivery of components such as fish oil (Duan et al., 2011).

Simple homogenization or mechanical stirring are one of the cheapest and lower-energy consuming methods that have shown to achieve acceptable results in emulsion formation (Alvarez et al., 2014; Buxmann et al., 2010). However, in order for this method to be successful, the use of a wall material with great emulsifying properties is necessary.

High-pressure homogenization is a process that could be added after simple homogenization to form more stable and homogeneous emulsions with particle sizes closer to or in the nano range (Salminen et al., 2014).

Ultrasonic homogenization has also been used in combination with mechanical stirring. Frede et al. (2014) found that ultrasonic homogenization of lutein-loaded β-lactoglobulin particles produced stable emulsions with high resistance to creaming.

Another processing technology that has not been extensively used is emulsification by membrane. Ramakrishnan et al. (2013) found that the inclusion of membrane emulsification as a first step to form microcapsules can increase the encapsulation efficiency of whey proteins/fish oil microcapsules, compared to using mechanical stirring only. This result was attributed to the smaller particle size obtained with membrane emulsification in comparison with mechanical stirring; additionally, the particles formed were rounder with a smoother surface and lacked inner oil vacuoles.

2.5 Whole foods matrices and their potential to encapsulate and deliver bioactive compounds

2.5.1 Eggs

2.5.1.1 Fortified table eggs: The dietary manipulation approach

One current approach to produce *n*-3 LC-PUFAs fortified eggs is to focus on dietary manipulation of the hen's feed (Moghadasian, 2008; Coorey et al., 2015; Lemahieu et al., 2015). Dietary manipulation of the hen's feed has been widely studied and nowadays is one of the leading research studies to assess its impact on the nutritional content of table eggs. Hen's diet is usually fortified with n-3 fatty acids from different sources such as fish oil, chia seed (*Salvia*

hispanica), and flaxseed (*Linum usitatissimum*) (Coorey et al., 2015; Arantes et al., 2009). This approach has produced egg with higher content (two to three-fold) of *n*-3 fatty acids mostly in the form of ALA.

Coorey et al. (2015) obtained the highest levels of ALA (321 mg/50 g yolk) in table eggs after two weeks of supplementing the hen's feed with 30% chia flour; in comparison, eggs obtained from regular diet hens showed a content of 18 mg/50 g egg yolk). Additionally, ALA content in eggs from the 20% flaxseed supplemented diet were higher than those from diets supplemented with 30% flaxseed after the second week of feeding. The negative effect of the increased flaxseed concentration was also observed in the hen's productivity and was attributed to a laxative effect in hens resulting in a lower nutrient absorption. In the same study, it was observed that *n*-3 LC-PUFAs, EPA and DHA, were present in eggs only when hen's feed was supplemented with fish oil, showing the limitations of hen's physiology to convert ALA into long-chain PUFAs. Moreover, the EPA+DHA absorption efficiency was very low; considering a concentration of about 45 mg/50 g egg yolk after 15 g of fish oil feeding.

Dietary manipulation has also shown positive results in the lipid proportions in egg yolk from quail (*Coturnix coturnix japonica*). In a study where quails were fed with 5% flaxseed (Arantes et al., 2009), a significant increase of about 8-fold in the content of ALA was obtained. The amounts of DPA and DHA also presented a significant increase, changing from 0.08 and 0.37%, respectively, in the control to 0.20 and 0.93% in the supplemented diet. Overall, the increase in the *n*-3 PUFAS content in the egg yolk resulted in a modification in the n-6:n-3 ratio from the native 21.3 to 4.52; which is a better value from the human nutrition point of view.

Although dietary manipulation has increased the levels of alpha-linoleic acid (ALA) in the egg yolk, the human body has a limited capacity (5-10%) to convert ALA into its bioactive forms EPA and DHA. Other issues associated with this approach are the high cost of production, high variation of the level of *n*-3 PUFAs in egg yolk, fishy taste in the final product, and lower shelf life (McClements et al., 2007).

2.5.1.2 Nutritionally enhanced egg products

A sector of the population remains concerned about the high cholesterol and saturated fat intake related to the consumption of eggs. Therefore, some research studies have been conducted to develop egg products with low or non-cholesterol content but with high *n*-3 LC-PUFAs levels.

Kasiss et al. (2010) developed a nutritionally enhanced egg product that matched the whole egg on proximate composition, with the advantage of replacing most cholesterol and saturated fat with *n*-3 PUFAs. However, the removal of egg yolk reduced the protein and mineral content in the final product. The sensory attributes and storage stability of this product was not studied.

2.5.2 Technologies applied to improve egg yolk emulsifying properties

From the information presented in section 1.5.1 and 1.5.2, it can be assumed that the emulsifying capacity is one of the most important characteristics that an encapsulating material should present. Increasing this capacity in an already excellent emulsifier like egg yolk, could lead to enhance its payload capacity, encapsulation efficiency, and stability of emulsions under harsher processing and storage conditions.

2.5.2.1 Enzymatic modification

The most common enzymes used to enhance the functional properties of egg yolk belong to the phospholipase family (Figure 2.3). To begin with, phospholipase A_1 (PLA_1) from *Thermomyces lanuginosus*, usually expressed in *Aspergillus Oryzae*, is used to improve egg yolk properties such as emulsifying and foaming capacity, protein solubility, and thermal stability. PLA_1 cleavages the ester bond at the sn-1 position of the triglycerides and converts phospholipids into lysophospholipids. It has been shown that treatment with PLA_1 improves the emulsifying and foaming capacity of egg yolk and its fractions, granules and plasma. One of the proposed mechanisms is the alteration of the LDL structure and reduction of the interfacial tension to a greater extend; changes that could significantly increase the molecular flexibility of LDL apoproteins, allowing for efficient absorption at the oil/water interface (Jin et al., 2013). The enzymatic treatment of egg yolk with PLA_1 has also shown an improvement of its thermal stability by increasing the first viscosity maximum from 79 to 97 °C (Jaekel and Ternes, 2009). These authors suggested that the action of phospholipases on the egg yolk phospholipids causes changes that interfere with the interactions between LDL and livetins during heat treatment, thus causing an impact on the first viscosity maximum. Among these changes a “shielding” effect of lyso-phosphatidylcholine (LPC) was considered; this effect is explained by the hydrophobic interaction of LPC and the hydrophobic core of LDL, forming a firm complex that blocks the interaction of LDL with denatured livetins (Mine, 1997).

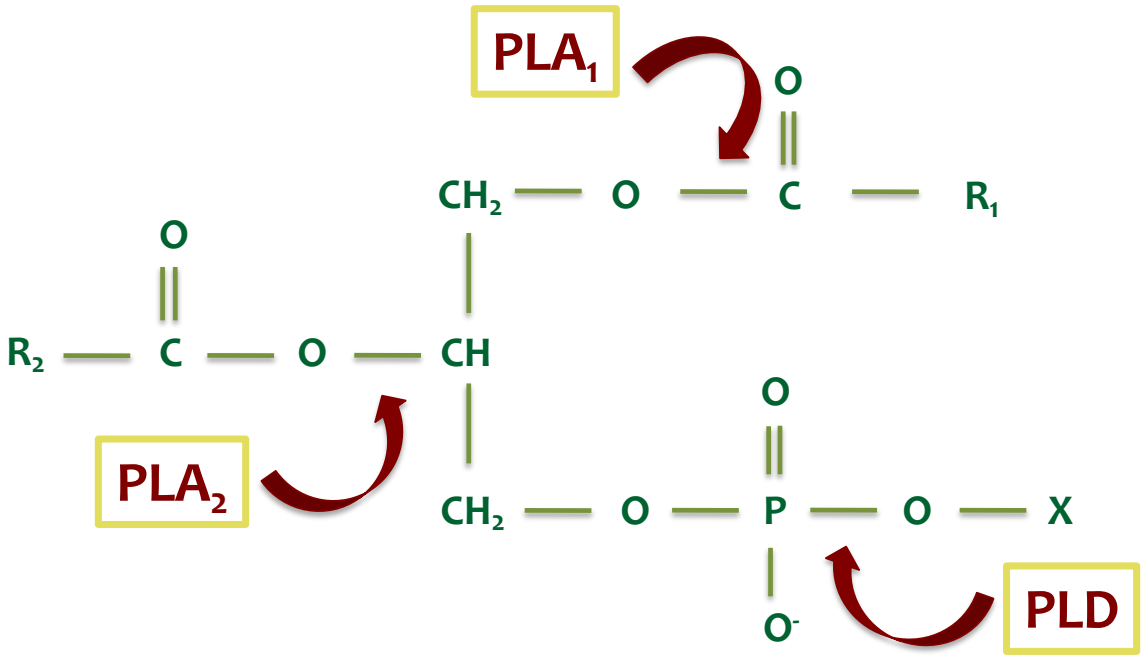

Figure 2.3 Phospholipase family and their cleavage sites.

Another widely tested enzyme, Phospholipase A_2 (PLA_2), catalyzes cleavage of the acyl group at the sn-2 position of the phospholipid molecules. This change has shown to improve heat stability of egg yolk emulsions (Dutilh and Groger, 1981). The interaction of phospholipids and proteins due to changed hydrophobicity of the lyso-phospholipids formed during incubation was reported responsible for the enhanced heat stability. The effect of PLA_2 in egg yolk fractions, plasma and granules, after spray drying has been also studied. Strixner et al. (2013) found that plasma treated with PLA_2 displayed better heat stability by preserving most of its native gelling properties after spray drying at an outlet temperature of 90 °C, in comparison with non-treated plasma, which lost its multistage gelling behavior at the same conditions. Additionally, the interfacial tension of modified plasma showed lower values than its native stage suggesting improved emulsifying properties. Furthermore, PLA_2-treated granules showed improved solubility and gel formation at lower temperatures than its native counterpart.

Finally, phospholipase D (PLD) has also been reported to increase the heat stability of egg yolk. This enzyme hydrolyses the phosphate ester group of phospholipids forming choline and phosphatidic acid from egg yolk lecithin. PLD can also cause a transphophatidylation with

other polar groups producing phospholipids with modified head groups. It has been shown that the activity of PLD increases in the presence of Ca^{+2} and phosphatidic acid, therefore, the activity rate of the enzyme in whole egg yolk as substrate is initially low: the degradation of phosphatidylcholine was negligible during the first hour of incubation (Buxmann et al., 2010). In the same study, an increased thermal stability of the egg yolk emulsions was also observed. This effect was attributed to a possible shielding effect of the phosphatidic acid due to its modified charge (negative) compared to that of phosphatidylcholine and phosphatidylethanolamine originally present in egg yolk.

2.5.2.2 Fractions separation

Separating the egg yolk two main fractions (granules and plasma) is a relatively easy task; a 1:1 dilution with 0.17 M NaCl solution followed by centrifugation for 45 min at 10000 x *g* (McBee and Cotterill, 1979). The fractions have been studied separately since the particular composition of each allows them to have different functional properties.

LDL has shown to be the main contributor to the egg yolk emulsifying capacity (Anton et al., 2003). During mechanical emulsification, the original spherical conformation of LDL is broken-up at the oil droplet surface thus allowing neutral lipids to diffuse into the oil phase (Martinet et al., 2003). While the granule fraction has a slightly lower apparent viscosity than egg yolk, it was reported that after phosvitin depletion, the leftover granules displayed a higher apparent viscosity than whole granules and egg yolk. This observation was attributed to the higher lipid and protein content as well as the denatured original structure of the phosvitin-depleted granules (Chalamaiah et al., 2018). In addition, the same study reported a higher emulsifying activity index of leftover granules in comparison with whole granules, when tested at a concentration of 0.5%. At the same conditions, leftover granules showed no significant differences in emulsifying activity index compared to egg yolk.

2.5.3 Other food matrices used for encapsulation of lipophilic compounds

Although several studies report the use of food-grade encapsulating materials and emulsions, to the best of our knowledge, there are very few reports on the efficiency of using a whole food matrix. The emulsifying capacity inherent to some food matrices confers them the ability to encapsulate and deliver lipophilic ingredients. For instance, in a clinical trial using 6 healthy

subjects, it was found that a dairy beverage, used as carrier of canola oil, produced triglyceride levels in blood similar to those of using sodium caseinate and whey protein isolate to pre-encapsulate the oil (Augustin et al., 2014).

Furthermore, hydrolyzed rice glutelin has also been used to emulsify corn oil. Xu et al. (2017), used different polysaccharides to enhance the emulsion stability of hydrolyzed rice glutelin-coated corn oil. They observe that the most pH-stable emulsions were obtained when using pectin and the most salt- and thermal-stable emulsion where obtained for those adding xanthan gum.

The importance of using food matrixes to encapsulate and deliver lipophilic bioactive components could be linked to a reduction in production costs at industrial scale. Moreover, it is important to study the effectiveness of the food matrix to protect the bioactive compounds throughout processing and shelf-life of the final product.

2.6 Impact of nutritional enhancement on the consumer perception and acceptability of food products

2.6.1 Determining the consumer acceptability of food products

The final, and probably most important, characteristic to consider when developing a delivery system with potential food applications is the consumer perception and acceptance of the final food product. These delivery systems should not only have proven an excellent stability during the encapsulation of the compound but should also guarantee stability during processing conditions and storage of the final food product. The initial sensory characteristics of the delivery system should be taken into account since they will have an impact on the sensory quality of the final product to certain extend.

Several quantitative and qualitative techniques have been developed to assess the sensory characteristics of a product. There are four major characteristics that are considered as part of a sensory test: Appearance, aroma, taste, and texture. The perception of these characteristics in a food product depends on how the food components interact with the sensory receptors in the human body (McClements, 2016).

Sensory evaluation is an integration of techniques to measure the response of the human senses to foods; it aims to minimize biasing effects of brands and other information that could potentially influence the consumer perception (Lawless and Heymann, 2010). The sensory

characteristics of a product are commonly evaluated using a trained panel. The training is usually conducted using specific descriptors for the product at different concentration. This kind of test seeks for panelists that have great sensory acuity and show reproducibility of their results.

Nevertheless, consumer acceptance can be assessed using untrained panelists. These panelists, however, are usually required to be regular consumers of the product to evaluate. An affective test is used for this purpose and it describes how well a product is liked or which product is preferred between two or more choices. The 9-point hedonic scale (Sjostrom, 1954) is the most used scale to assess the degree of liking or disliking of a product. The preference can be determined by using a choice (for two products) or a ranking test (for three or more) (Lawless and Heymann, 2010).

2.6.2 Consumer acceptability of *n*-3 PUFAS-enriched food products

Consumer acceptability is a crucial outcome for the development of enriched egg products. Some egg products that provide low fat and high protein concentrations are usually not well accepted by the consumer when compared to the whole egg (Leutzinger et al., 1977). In a study conducted by Hayat et al. (2014) it was found that nutrient-enriched eggs, produced by feed manipulation, were accepted by the consumers showing similar overall quality with regular eggs; significant differences were obtained only for the egg yolk color. However, the number of panelists (n= 10) was below the minimum acceptable for a consumer acceptance analysis. In a more complete sensory study, Coorey et al. (2015), reported no significant changes in consumer preference of table eggs from hens with supplemented diets (30% chia flour) in comparison with the control eggs. Nevertheless, they observed a decrease in the overall consumer acceptability when supplementing the diets with 30% flaxseed flour and 40% chia seed flour.

Another food commodity that has been extensively tested for *n*-3 PUFAs incorporation is dairy products. The effect of the addition of 500 mg *n*-3 fatty acids from an algae oil emulsion to strawberry flavored yogurt was studied by Chee et al. (2005). The addition of *n*-3 fatty acids was tested before and after homogenization. Trained panelists detected a strong fishy flavor for both treatments; however, untrained consumer panelists rated the treatments as they did the control (without added *n*-3).

2.7 Conclusions

The information collected and presented in this review helped develop and support our hypothesis of egg yolk as a promising carrier of *n*-3 LC-PUFAs. Egg yolk presents most of the desired characteristics in a delivery system, with potential improvement through enzymatic modifications and the use of polysaccharides as an extra barrier to protect unstable components from the environment. In the following chapters of this thesis we show the different stages of our research that assessed the behavior of egg yolk during the development of a food product with higher content of EPA and DHA that is accepted by the final consumer.

CHAPTER 3: Encapsulation of long chain *n*-3 polyunsaturated fatty acids using egg yolk

3.1 Abstract

Egg yolk is well known for its excellent emulsifying property. In this research, egg yolk was used as the encapsulating matrix to prevent the oxidation of *n*-3 long chain polyunsaturated fatty acids. A 2x2x5 Complete Block Design with three replications was used. Two levels of fish oil concentrates (1% and 5%), and two levels of esterification type (triglycerides or ethyl esters) of eicosapentaenoic/docosahexaenoic fatty acids were used. Time was considered a fixed factor with 5 levels. Emulsion was prepared by homogenization. The resulting samples were stored up to 4 weeks at 4–6 °C, sampled weekly, and analyzed for particle size and distribution, encapsulation efficiency, and surface oil. The oxidative stability of the emulsions was evaluated before and after cooking. The addition of triglycerides resulted in a larger average particle size (234±12.4 nm). All emulsions achieved 100% encapsulation efficiency and showed no significant change in the surface oil concentration during storage. After four weeks of storage, the concentration of eicosapentaenoic + docosahexaenoic fatty acids in non-encapsulated fish oil triglycerides and ethyl esters decreased by 20.3% and 14.7%, respectively; while the content in emulsions did not vary. Additionally, no peroxides or propanal formation was detected in raw emulsions over the storage period. Propanal formation was negligible in cooked samples and the peroxide value showed no differences between the egg yolk control and the emulsions. Therefore, egg yolk showed to be an efficient encapsulating food matrix that protects *n*-3 polyunsaturated fatty acids against oxidation and degradation.

3.2 Introduction

The annual egg per capita consumption in Canada reached 19.9 dozen in 2016, representing an increase of 3.5 percent annually in the last five years. Approximately 73% accounts for table eggs, while the remaining 27% is further processed (Agriculture and Agri-Food Canada, 2017). The egg yolk is highly valuable to the food industry due to its emulsifying and gelling capacity, coloring, and antioxidant properties. It also provides nutrients with high biological value such as proteins, lipids, vitamins and minerals (Huopalahti et al., 2007). The egg yolk is constituted of two main fractions: the micro-fraction, also called granules, consist of high-density lipoproteins (HDL) and phosvitin linked by phosphocalcic bridges, and the nano-fraction (plasma) which is mainly composed by low-density lipoproteins (LDL) (Burley and Cook, 1961; Anton and Gandemer, 1997). *n*-3 long chain polyunsaturated fatty acids (*n*-3 LC-PUFAs), in the form of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can be found in egg yolk and are particularly important due to their associated benefits to human health (Huopalahti et al., 2007). However, eggs are not a significant source of EPA and DHA since their natural content is lower than the recommended daily intake (RDI) by the American Heart Association (250 mg per day for healthy adults and up to 4g for people with heart conditions). Thus, there is a need to seek out alternatives to increase the content of these nutrients in eggs. One current approach to produce *n*-3 LC-PUFAs fortified eggs is to focus on dietary manipulation of the hen's feed (Moghadasian, 2008; Coorey et al., 2015; Lemahieu et al., 2015). This approach has increased the levels of alpha-linoleic acid (ALA) in the egg yolk. However, the human body has a limited capacity to convert ALA into EPA and DHA (5-10%). Other issues associated with this approach are the high cost of production, low efficiency of transformation, high variation of the level of *n*-3 fatty acids in egg yolk, fishy taste in the final product, and shorter shelf life (McClements et al, 2007).

Another strategy to fortify food products with n-3 PUFAS is to use micro and nano-capsules than can act as the delivery system for those nutrients (Kassis et al., 2010; Duan et al., 2010; Matalanis et al., 2011; Gulotta et al., 2014). The ideal *n*-3 carrier is food grade, has high chemical compatibility with different food matrixes, is cost-effective to produce, has high payload and encapsulation efficiency, a high emulsion stability, small particle size, and protects against oxidation (Gulotta et al., 2014; Ramakrishnan et al., 2013). This last characteristic is of special importance since the multiple double bonds in the *n*-3 LC-PUFAs structure make them highly susceptible to oxidation, which decreases the shelf life, consumer acceptability,

functionality, and nutritional value (Arab-Tehrany et al., 2012). Moreover, this reaction generates secondary oxidation products that have been linked with aging, mutagenesis, and carcinogenesis.

Previous studies of *n*-3 fatty acids rely on encapsulation using isolated wall materials, such as proteins, polysaccharides, and lipids. Nevertheless, using these wall materials usually present issues such as low encapsulation efficiency and low stability of the emulsions (Chung et al., 2010; Alvarez et al., 2014). To the best of our knowledge, little research has been conducted using whole food systems to encapsulate and protect bioactive components, hence, the importance of this study. We hypothesize that egg yolk is a suitable carrier to encapsulate *n*-3 LC-PUFAs. Thus, we focused on using a processing approach to develop an oxidative stable egg yolk product that provides at least 100% of the recommended daily intake of EPA and DHA in one serving (30g). This approach may overcome one of the dietary manipulation issues by allowing for better control of the concentration of EPA/DHA in the final product. In addition, developing a nutritionally enhanced egg product will add value to the egg industry.

3.3 Materials and methods

3.3.1 Materials

Fresh white shell eggs were collected from the Alberta Poultry Research Centre (Edmonton, AB, Canada) and used within 3 days of storage at 4–6 °C. AlaskOmega Products provided the organic fish oil concentrates from Alaska Pollock (*Gadus chalcogrammus*) containing ≥83% of EPA+DHA as triglycerides (TG) or ethyl esters (EE). All the reagents used for the chemical analyses were HPLC grade obtained from Sigma-Aldrich (Oakville, ON, Canada).

3.3.2 Sample preparation

3.3.2.1 Raw emulsions

Egg yolks were separated manually from the whites and rolled on Whatman no.1 paper to eliminate the albumen residues. The vitelline membrane was then punctured with a spatula and the egg yolk content was collected in a container placed in an ice bath. Fish oil was then added to the egg yolk to obtain four different treatments: 1 and 5% (w/w) EPA/DHA triglycerides (1%TG and 5%TG, respectively), and 1 and 5% (w/w) EPA/DHA ethyl esters (1%EE and 5%EE, respectively).

Approximately 100 g samples (egg yolk + fish oil) were homogenized in an IKA T25 Ultra Turrax (IKA Works Inc., Wilmington, USA) at 24000 rpm for 4 min then divided into five individual plastic vials and sealed with parafilm; an aliquot of the whole sample was used to determine moisture content. Samples were then pasteurized at 61.1 °C for 3.5 min according to Canada Processed Egg Regulations, in order to simulate industrial processing conditions. Following pasteurization, the samples were stored for 4 weeks at 4–6 °C and analyzed weekly. Raw egg yolk (egg yolk), non-encapsulated fish oil concentrates as triglycerides (FO-TG), and ethyl esters (FO-EE) were processed and stored at the same conditions as the treatments and used as controls. The emulsions were characterized according to their particle size distribution (PSD), encapsulation efficiency (EEf), surface oil during storage, and oxidative stability. In addition, the oxidative stability of the emulsions after cooking was also evaluated.

3.3.2.2 Cooked emulsions

In order to test the stability under cooking conditions, about 2 g of the emulsions (including egg yolk) were fried in 4 g canola oil at 150–170 °C. Samples were fried for 75 s, then flipped and fried for 45 s more. Immediately after, the cooked sample was cut in four pieces, put into a headspace vial, and sealed for further headspace analysis. In addition, 10 g egg yolk and emulsions were cooked following the same procedure and used for lipid extraction to quantify peroxides.

3.3.3 Viscosity of the emulsions

The apparent viscosity, at 25 °C, of the emulsions containing and the control was determined by steady state shear measurements (0.1–100 s^{-1}) in a Modular Compact Rheometer 302 (Anton Paar, Graz, Austria) equipped with a 50 mm diameter parallel plate measuring tool, using a 1 mm gap.

3.3.4 Particle size and distribution

Samples were diluted 1:4 (w/w) with a 0.1% (w/w) sodium dodecyl sulfate solution, then vortexed for 30 s prior to analysis to avoid multiple scattering. The particle size (as given by the Sauter mean diameter, d_{32}) and distribution of the emulsions were measured by static light scattering using a Mastersizer 2000 (Malvern Instruments Ltd, Worcestershire, UK) set at a

refractive index of 1.4215 and 2000 rpm. Samples were added one drop at a time into the Mastersizer measurement chamber until an optimum obscuration rate between 10 and 20% was achieved.

3.3.5 Analysis of the emulsions by light microscopy

The emulsions were diluted 1:10 with distilled water, then a drop of the solution was placed in a microscope slide, covered with a glass slid, and let dry overnight on a desiccator. After drying, a drop of Oil Red O solution (0.5% in isopropanol) was added to the film to dye the lipids in the emulsion. The films were observed at 40x in a Zeiss Axio Scope A1 light microscope.

3.3.6 Encapsulation efficiency and surface oil during storage

The encapsulation efficiency was determined by quantifying the total and surface EPA+DHA in the particles, using the following formula:

$$EEf\ (\%) = [(\text{Total EPA+DHA} - \text{Surface EPA+DHA})/\text{Total EPA+DHA}] \times 100 \quad (1)$$

3.3.6.1 Total oil

To quantify the total EPA+DHA, total oil was first extracted using the method proposed by Bligh and Dyer (1959) with the following modifications: approximately 10 g of the sample previously adjusted to 80% moisture was mixed with 30 mL of a chloroform: methanol solution (1:2 v/v) and homogenized at 24000 rpm for 2 min, then added 10 mL of chloroform and homogenized at the same condition for 30 s. Immediately after, 5 mL of distilled water was added and homogenized for 30 s. The samples were vacuum filtered through Whatman No. 1 paper. The lipid remaining in the residue was recovered by rinsing with 15 mL chloroform. The filtrate was transferred to a separatory funnel, and after complete separation, the chloroform layer was recorded in a weighted glass vial and evaporated under nitrogen stream until constant weight.

3.3.6.2 Surface oil

The surface oil was quantified by hexane extraction as described by Alvarez et al. (2014) with slight modifications. About 2 g samples were mixed with 15 mL of hexane and stirred at 200 rpm for 15 min; the process was repeated twice. The hexane fractions were filtered using syringe

filters with a membrane pore size of 0.45 μm; the solvent was collected in a glass tube and evaporated under a nitrogen stream.

Once total and surface oil samples were obtained, the vials were capped with nitrogen, sealed tightly and stored at -20 °C for no more than 12 hours to prepare for methylation.

3.3.6.3 Methylation and EPA+DHA quantification

The methylation and quantification of EPA+DHA was conducted following the method developed by Joseph and Ackman (1992). Briefly, 25 mg (±0.1 mg) oil sample was placed in a tube containing 1 mg of internal standard then 1.5 mL 0.5 N methanolic NaOH were added and heated for 5 min at 100 °C. After cooling to room temperature, 2 mL BF_3 (12% v/v in methanol) were added then heated 30 min at 100 °C. The mixture was cooled to 30 – 40 °C followed by addition of 1 mL isooctane, vortexed for 30 s and immediately added with 5 mL saturated NaCl solution (36 g NaCL in 100 mL distilled water). After complete separation, the isooctane layer was transferred to a glass tube then the aqueous phase was extracted one more time with an additional 1 mL isooctane. The isooctane extracts were combined and concentrated to 1 mL under nitrogen stream. 23:0 methyl esters were used as internal standard for triglycerides samples. Ethyl ester samples did not require further methylation; therefore, samples were directly mixed with 1 mg 23:0 ethyl esters internal standard and dissolved in 1 mL isooctane.

For the surface oil samples, the amount of oil obtained was below 25 mg. Therefore, the weight of the oil was recorded, and the appropriate internal standard was directly added into the glass tube for methylation.

The area counts of EPA and DHA was quantified by injecting 1 μL of the corresponding methyl or ethyl ester samples into an Agilent 7890 Gas Chromatograph with 5975 Mass Selective Detector using MSD Chemstation software. The carrier gas was Helium 5.0 and the column was an Agilent HP-5 MS, 30m x 250μm x 0.25μm. The inlet temperature was set at 200°C and operated in splitless mode. The oven was set to 50°C for 5min, then increasing at 5°C/min until 320°C for 5 min. Total run time was 64 min. The mass spectrum was scanned from 40-500 m/z.

EPA and DHA in methyl esters samples were quantified using the following formula:

$$\text{EPA or DHA mg/g oil} = [(A_X \times W_{IS} \times CF_X) / (A_{IS} \times W_S \times 1.04)] \times 1000 \quad (2)$$

where A_X is the area counts of EPA or DHA; A_{IS} is the area counts of internal standard; CF_X is the theoretical detector correction factor, 0.99 for EPA and 0.97 for DHA; W_{IS} is the weight of internal standard added to the sample, mg; W_S is the weight of the sample, mg; and 1.04 is the factor necessary to express results as mg fatty acid / g oil (rather than as methyl esters).

EPA and DHA in ethyl esters samples were quantified using the following formula:

$$\text{EPA or DHA mg/goil} = [(A_X \times W_{IS} \times CF_X) / (A_{IS} \times W_S \times 1.08)] \times 1000 \quad (3)$$

where terms are the same as in (2), except use 1.08 as the factor necessary to express results as mg fatty acid/g oil (rather than as ethyl esters).

3.3.6.4 Surface oil changes during storage

The concentration of surface EPA+DHA of the emulsions was measured weekly to assess the stability of the particles.

3.3.7 Peroxide value in raw and cooked emulsions

The quantification of peroxides was conducted in the whole raw emulsion and the lipids extracted from cooked samples. Canola oil cooked for 2 min at 150 °C was used as blank for peroxide determination in cooked samples.

The peroxide value (POV) was measured following the AOAC official method 965.33, which measures the iodine liberated from potassium iodide by a peroxide in the presence of acetic acid, using sodium thiosulfate as the titrant. A blank determination of the reagents was conducted every day before starting the analyses. About 5 g of the sample was combined with 30 mL of solvent containing acetic acid and chloroform (3:2, v/v), and homogenized at 24000 rpm for 30 s. 0.5 mL of a saturated KI solution was then added, let stand for 1 min with occasional shaking, and then 30 mL of distilled water were added. The final mix was titrated with 0.1 M $Na_2S_2O_3$ solution while vigorously shaking until the yellow was almost disappeared. Then, 0.5 mL of a 1% starch solution was added, and titration continued, until the blue disappeared.

The peroxide value was calculated using the following formula:

$$\text{meq peroxide/kg oil} = (\text{mL } Na_2S_2O_3 \times \text{molarity } Na_2S_2O_3 \times 1000)/\text{g test portion} \quad (4)$$

3.3.8 Propanal content in raw and cooked emulsions

Propanal formation in raw and cooked emulsions was monitored by headspace chromatography (REF). About 2 g of the raw emulsion or 2 g of the omelet were placed into a 20 mL headspace vial, sealed and heated at 60 °C for 30 min, followed by 30 min of equilibration time at room temperature. The headspace was analyzed using a capillary gas chromatograph with a Restek Stabilwax (Crossbond Carbowax) column, measuring 30 meters in length, with a 0.53 mm internal diameter, and a 0.5 µm df. The carrier gas was helium and the oven were set to 45°C; the total run time was 2 min. The detector and injector temperature were 250°C. Propanal content was calculated using a standard curve (r^2= 0.99) built with 0, 3, 6, 9, 12, and 15 µg propanal (98% purity) in water and expressed in µg propanal/g sample.

3.3.9 Statistical analysis

A 2x2 Complete Block Design with three replications was used. The independent factors were the percentage of fish oil concentrates added (1% and 5%), and the EPA+DHA type in the fish oil concentrates (triglycerides or ethyl esters). Time was considered a fixed factor with 5 levels. Results were expressed as the mean and the standard error. A multiple analysis of variance (MANOVA) was used to find significant factors over time and a one-way analysis of variance (ANOVA) was used to analyze differences at one given time. All the statistics were conducted using the statistical package R version 3.3.2 in R studio version 1.0.136 (R Core Team, 2016). Significant differences of the mean values were considered at $p < 0.05$ using Tukey test.

3.4 Results and discussion

In this study, we used egg yolk to encapsulate *n*-3 LC-PUFAs. We characterized and evaluated the stability of three controls and four encapsulated egg yolk samples and studied the stability under storage and cooking. Adding 1% fish oil was aimed to achieve 100% of the EPA+DHA RDI for a healthy adult in one egg yolk portion (30 g) while adding 5% was chosen for the population requiring higher RDI.

3.4.1 Viscosity and imaging of emulsions

The apparent viscosity of the samples changed depending on the carrier and the processing type. All samples, regardless of the treatment, showed a shear-thinning behavior at increasing shear

rate (Figure 3.1). However, emulsions containing 5% fish oil as TG or EE showed a higher viscosity at shear rates above 1 1/s. An increase in the apparent viscosity has been observed during the production of oil-in-water emulsions where egg yolk is the main emulsifier. This behavior results from an increase of the droplet concentration at higher disperse volume fraction (McClements, 2016).

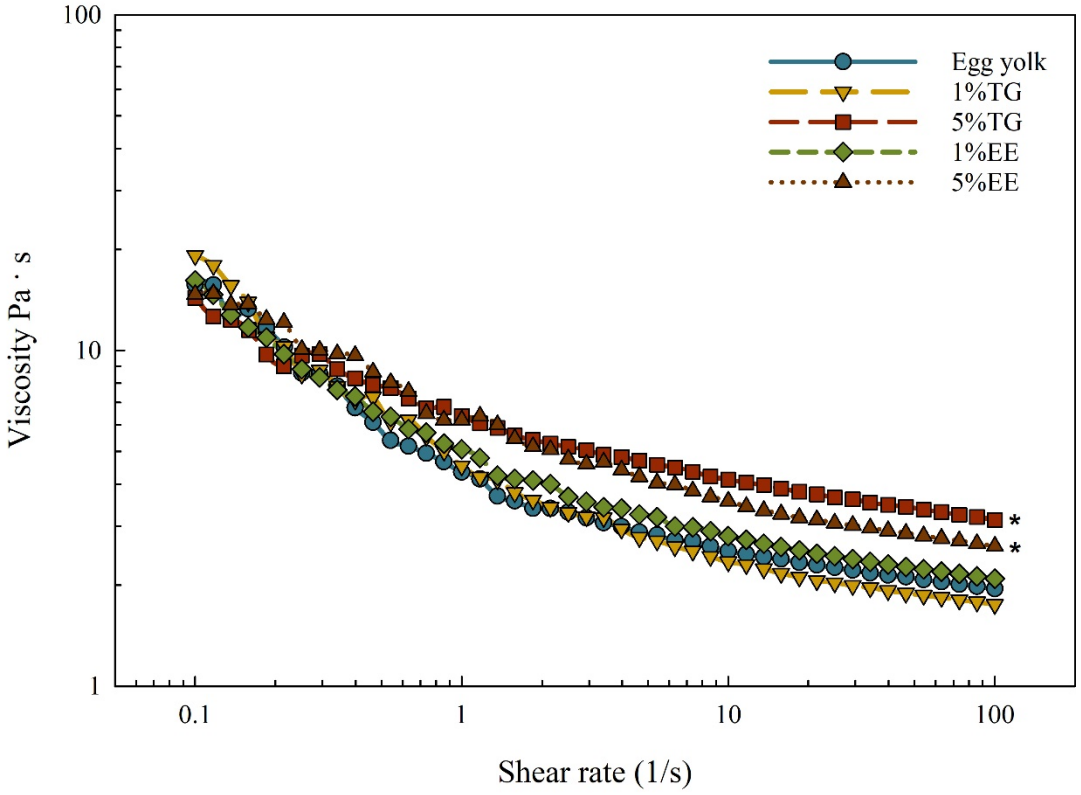

Figure 3.1 Apparent viscosity of egg yolk emulsions containing 1 or 5% fish oil triglycerides (1%TG and 5%TG) or ethyl esters (1%EE and 5%EE) and the control (Egg yolk). Significant differences at α= 0.05 between the treatment and the control are indicated by *, for shear rates above 1 1/s.

Figure 3.2 shows images observed at a 40x magnification of the egg yolk control (a) and 5%TG (b) emulsions. The total lipids (colored in red) in the films seems to be evenly distributed; however, no clear difference in the droplet concentration is appreciated probably due to the low concentration of added oil.

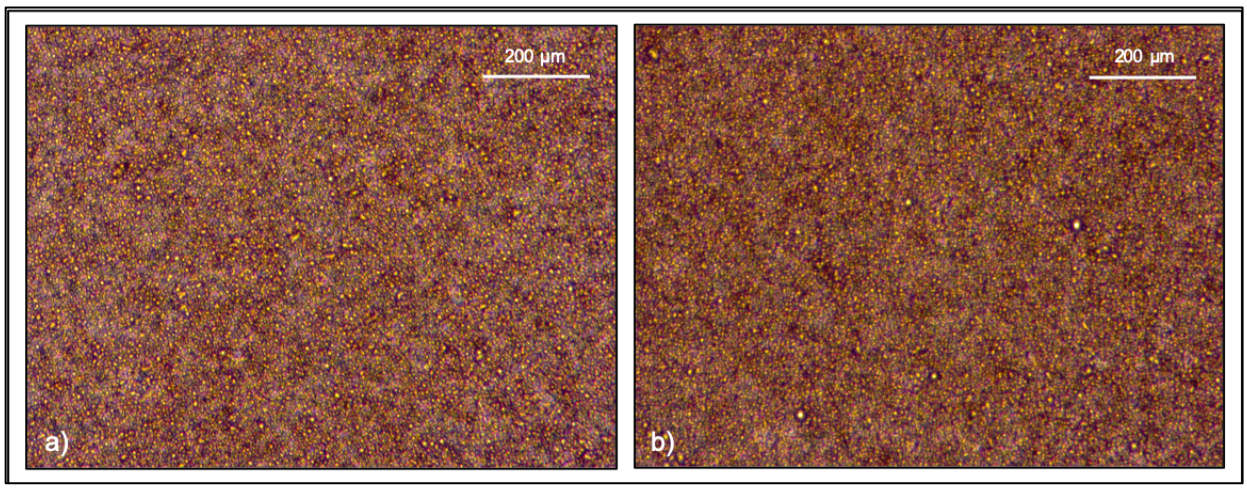

Figure 3.2 Images of egg yolk control (a) and 5%TG (b) emulsion films at 40x magnitude using light microscopy and Oil O Red for lipid staining.

3.4.2 Particle size and distribution

The Sauter mean diameter (d_{32}) was used to determine the effect of the concentration and type of fish oil on the particle size and distribution of egg yolk emulsion. Changes over time were measured to assess the physical stability of the samples during storage. The fish oil concentration and type had a significant effect on particle size ($p < 0.0001$; Figure 3.3). We observed that samples containing EPA+DHA ethyl esters presented the smallest particle size, regardless the concentration, and were not significantly different from egg yolk. In comparison, adding TG increased the particle size of raw egg yolk and emulsions containing EE, and showed a positive correlation with the concentration. Overall, 5%TG had an average size of 234±12.4 nm, being the largest observed among all treatments including the egg yolk control. On the other hand, no significant changes ($\alpha = 0.05$) in the particle size were observed during the storage time evaluated.

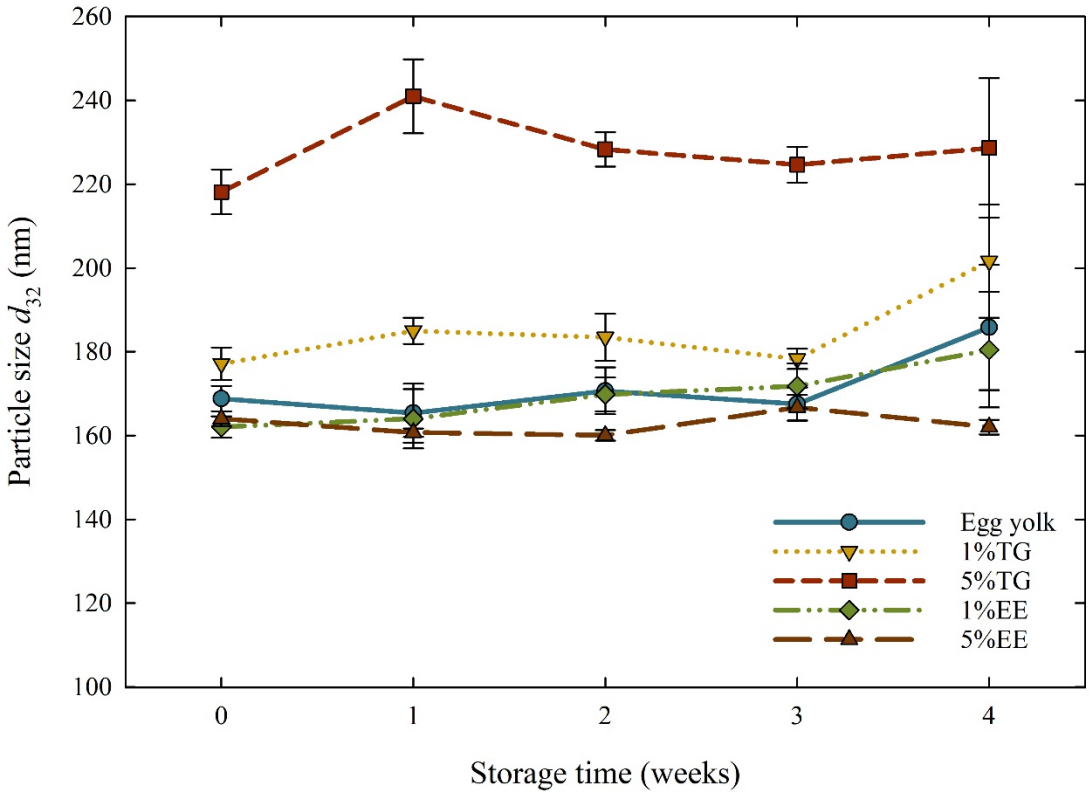

Figure 3.3 Average particle size as given by the Sauter mean diameter (d_{32}) of egg yolk control (Egg yolk) and emulsions (1%TG, 5%TG, 1%EE, 5% EE) over 4 weeks of storage at 4–6 °C. Each point corresponds to the mean ± standard error of three replications. Treatments and the control did not show significant changes ($p > 0.05$) in the particle size over time.

Ideally, micro and nano-capsules should have small particle size with a narrow monomodal distribution because it might affect the payload and stability of the emulsions (Gulotta et al., 2014; Frede et al., 2014; McClements, 2017). Nevertheless, raw egg yolk was known to present a bimodal distribution. Therefore, an analysis of the particle size distribution was also conducted (Figure 3.4) to assess the effect of using EPA/DHA triglycerides or ethyl esters. The concentration of fish oil did not affect the PSD of the samples; also, no changes were observed over time (*data not shown*). Therefore, two samples (5%TG, and 5%EE) freshly prepared were selected to represent the PSD in comparison with egg yolk. The egg yolk presented a pH ranging from 5.62 to 5.75. Under these conditions, two peaks were displayed in the distribution graph (bimodal curve). The first peak observed in the nano-size region was related to plasma, composed mainly of LDL assembles. The small particle size reported was 40 nm, corresponding to the LDL assembles (16-60 nm, Cook and Martin, 1969). The second peak

corresponded to granules (1-8 μm). The addition of fish oil containing ethyl esters altered the pattern of these two native fractions, increasing the proportion of plasma while decreasing the granule fraction. Conversely, when fish oil triglycerides were added, the volume% of granule aggregates increased to 4.61%, while that of plasma decreased by 0.8% in comparison with the distribution in raw egg yolk.

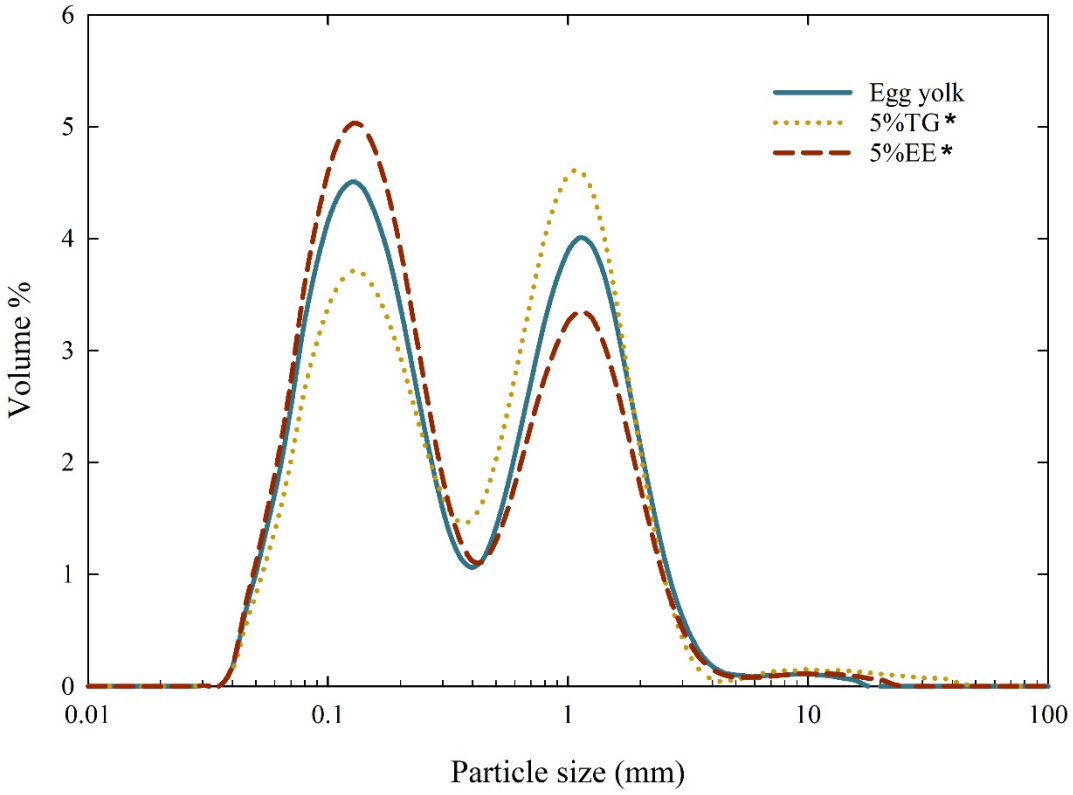

Figure 3.4 Particle size distribution of egg yolk control (Egg yolk), 5% fish oil triglycerides (5%TG), and 5% fish oil ethyl esters (5%EE) emulsions at week 0. The graph is an average of three replications measured in duplicates. * indicates significant differences ($p < 0.05$) with the egg yolk control.

Our results suggested that granules can encapsulate more EPA+DHA when they are in triglyceride form than ethyl ester. In order to prove this assumption, egg yolk was fractionated into plasma and granules and the EPA+DHA in both fractions were quantified. It was found that the total oil and EPA+DHA in granules increased when fish oil triglycerides were used, in comparison with emulsions containing fish oil ethyl esters (Table 3.1). These results proved that granules could encapsulate more EPA+DHA as triglycerides than as ethyl esters, resulting in the observed volume increase in the micro-fraction and the larger average particle size.

Table 3.0 Total oil and EPA+DHA content in plasma and granules from egg yolk emulsions containing 5% fish oil triglycerides or ethyl esters (5%TG and 5%EE, respectively). Different letters within column indicates significant differences at $\alpha = 0.05$.

Treatment	Total oil in plasma (%)	Total oil in granules (%)	EPA+DHA (%) in plasma	EPA+DHA (%) in granules
5%TG	90.2[a]	9.8[a]	80.4[a]	19.6[a]
5%EE	90.9[b]	9.1[b]	81.9[b]	18.1[b]

3.4.3 Encapsulation efficiency and surface oil changes during storage

The encapsulation efficiency is commonly measured to assess the efficiency of the wall material(s). The ideal microcapsules have high encapsulation efficiency and low or no surface oil during storage. This characteristic is of special importance for particles enclosing lipophilic compounds because the surface oil is more accessible to oxygen and therefore more prone to oxidation (Baik et al., 2004). For the purposes of this study, the EEf and surface oil during storage was measured considering the total: surface EPA+DHA ratio.

During the assessment of native *n*-3 LC-PUFAs in egg yolk, it was found that EPA was not naturally present. However, DHA was present at a concentration of 4.97 ± 0.37 mg/g egg yolk. This amount was deducted from the total quantification of DHA in the egg yolk: fish oil emulsions for further EEf assessment. For comparison purposes, the surface oil in the egg yolk control was also evaluated, but no DHA was detected, indicating that the DHA is naturally encapsulated within the egg yolk structures. Prior to starting EEf analyses, the EPA+DHA concentration in the standard fish oil concentrates were quantified, giving the result of 815.04±4.26 and 843.16±4.58 mg/g for FO-TG and FO-EE, respectively. The total EPA+DHA in the emulsions after homogenization at 24,000 rpm was not significantly different from the theoretical amount added (α= 0.05), suggesting this processing method as an effective alternative to hen's feed modification to develop EPA+DHA fortified egg yolk products.

Table 3.2 shows the EEf for each treatment. The concentration and type of fish oil had no influence on the EEf of the emulsions. Moreover, the EEf obtained for each level of the treatments was not significantly different from 100%.

Table 3.2 Encapsulation efficiency of egg yolk emulsions containing EPA+DHA as ethyl esters (EE) or triglycerides (TG). No significant differences were observed between the encapsulation efficiency (%) of samples and 100% evaluated at $\alpha = 0.05$.

Treatment	Encapsulation efficiency of EPA (%)	Encapsulation efficiency of DHA (%)	Total encapsulation efficiency (%)
1%TG	99.7	100.0	99.7
5%TG	99.5	99.8	99.7
1%EE	100.0	100.0	100.0
5%EE	100.0	100.0	100.0

Wang et al. (2014) achieved similar encapsulation efficiency of tuna oil in freeze-dried gelatin-sodium hexametaphosphate microcapsules produced by complex coacervation. Most studies have been conducted using different wall material combinations to encapsulate fish oil but most EEf determinations are made in powders obtained by different drying technologies (Liu et al., 2010; Anwar and Kunz, 2011; Wang et al., 2011; Wang et al., 2014; Alvarez et al., 2014). The advantage of our study is achieving a high EEf using a natural food matrix, which implicates lower cost of production.

The total and surface EPA+DHA were monitored during four weeks of storage under refrigeration conditions to assess stability. Total EPA+DHA in FO-TG and FO-EE was also monitored and used as negative controls. MANOVA analysis showed that there was no significant difference (α=0.05) in the surface EPA+DHA over time for each treatment.

For the egg yolk control, there was not surface DHA detectable over time. In addition, the original amount of EPA+DHA in the emulsions at week 0 remained without significant changes over time (*data not shown*). Figure 3.5 shows the surface EPA+DHA over four weeks of storage, as given by its concentration on the particle surface. The highest surface EPA+DHA mean was 0.14% and corresponded to the sample 5%TG at week 4. However, this amount was not significantly different from zero.

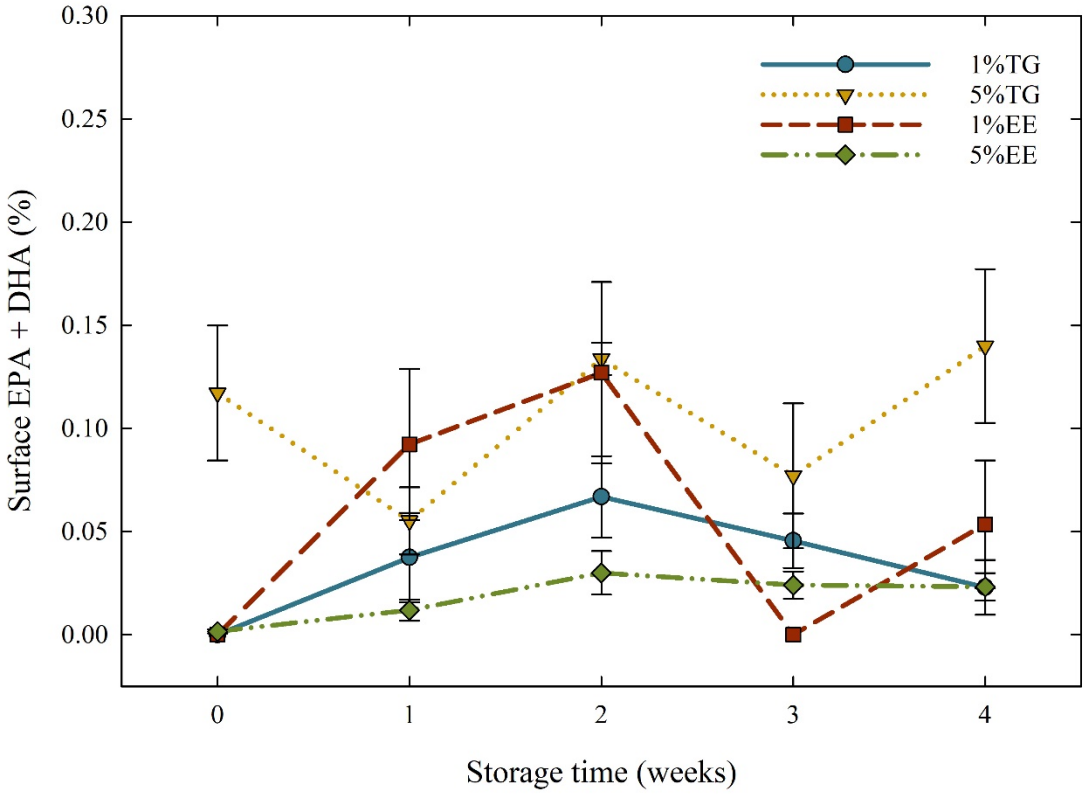

Figure 3.5 Percentage of surface EPA+DHA in egg yolk: fish oil emulsions over four weeks of storage at 4–6 °C. Each point corresponds to the mean ± standard error of three replications. The surface EPA+DHA percentages detected in treatments were not significantly different ($p > 0.05$) from 0% at any time point.

Conversely, the negative controls showed a significant decrease in EPA+DHA contents: 5.07% for triglycerides and 3.78% for ethyl esters, after homogenization and pasteurization (Figure 3.6). In addition, significant decreases were detected weekly, obtaining a total reduction of 20.32 and 14.74% for triglycerides and ethyl esters, respectively, after 4 weeks of storage.

It is well known that *n*-3 LC-PUFAs are highly unstable and oxidize easily in the presence of oxygen and high temperatures. Therefore, the EPA+DHA degradation in non-encapsulated fish oil was attributed to oxidation reactions catalyzed by processing conditions. The lack of significant differences within and among emulsions during storage indicates that egg yolk can encapsulate 1 or 5% of EPA+DHA without affecting its efficiency.

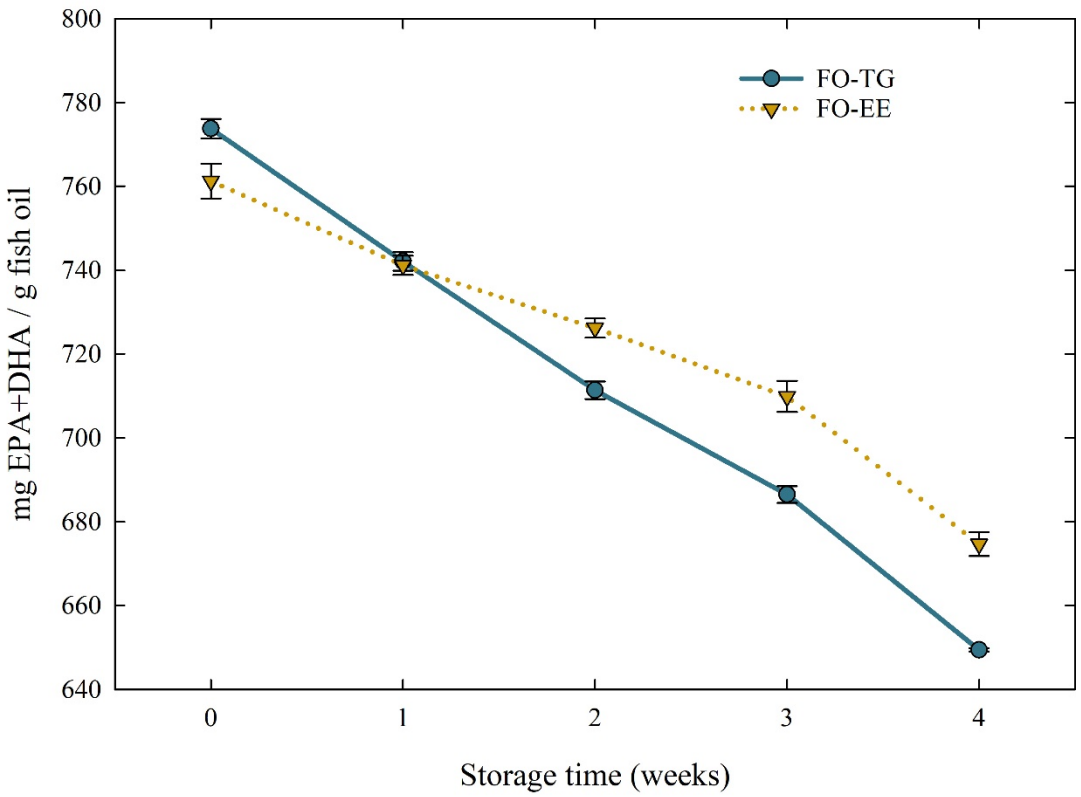

Figure 3.6 EPA+DHA degradation in fish oil triglycerides (FO-TG) and fish oil ethyl esters (FO-EE) over four weeks of storage at 4–6 °C. Each point corresponds to the mean ± standard error of three replications. The concentrations of EPA+DHA before processing were 815.04±4.26 and 843.16±4.58 mg/g for FO-TG and FO-EE, respectively.

3.4.4 Oxidative stability

The peroxide value is the most commonly used method to assess oxidation of oils, although the method is time consuming and measures unstable products, it also gives an indication of the first stage of oxidation of the product. Quantifying the propanal content is a high-cost method but it is important since propanal can be used as a specific marker for secondary oxidation of *n*-3 fatty acids. Therefore, results from these two analyses provided more details about the different stages in the oxidation of our samples.

3.4.4.1 Peroxide value

The peroxide value is an indicator of oxidation present as it specifies the content of oxygen as peroxide in a substance. Peroxides and similar products oxidize potassium iodide under acidic

condition and thus contribute to the peroxide value. The supplier reported a value of 0.5 meq/kg, to a maximum of 1.0 meq/kg, to comply with the quality standards of the oil. The POV of egg yolk and all the emulsions remained under 0.5 meq / kg over four weeks of storage. On the contrary, the POV increased significantly in FO-TG and FO-EE on week 0 after processing and kept increasing weekly, reaching a maximum of 202.7 ± 2.1 meq/kg for FO-TG and 156.2 ± 0.9 meq/kg for FO-EE on week 4 (Figure 3.7). Wang et al. (2011) observed a similar pattern for crude fish oil, reaching 350 meq/kg after 8 weeks of storage at 40°C. They also found that barley protein microcapsules decreased the POV of fish oil to 45 – 76 meq/kg. They attributed this oxidation to the exposure of the surface oil to oxygen, light and heat during processing, especially during spray drying. However, in our study, the emulsions were processed and store in the presence of light and oxygen, and pasteurization temperatures (61.1 °C, 3.5 minutes), without affecting the original POV of the oils.

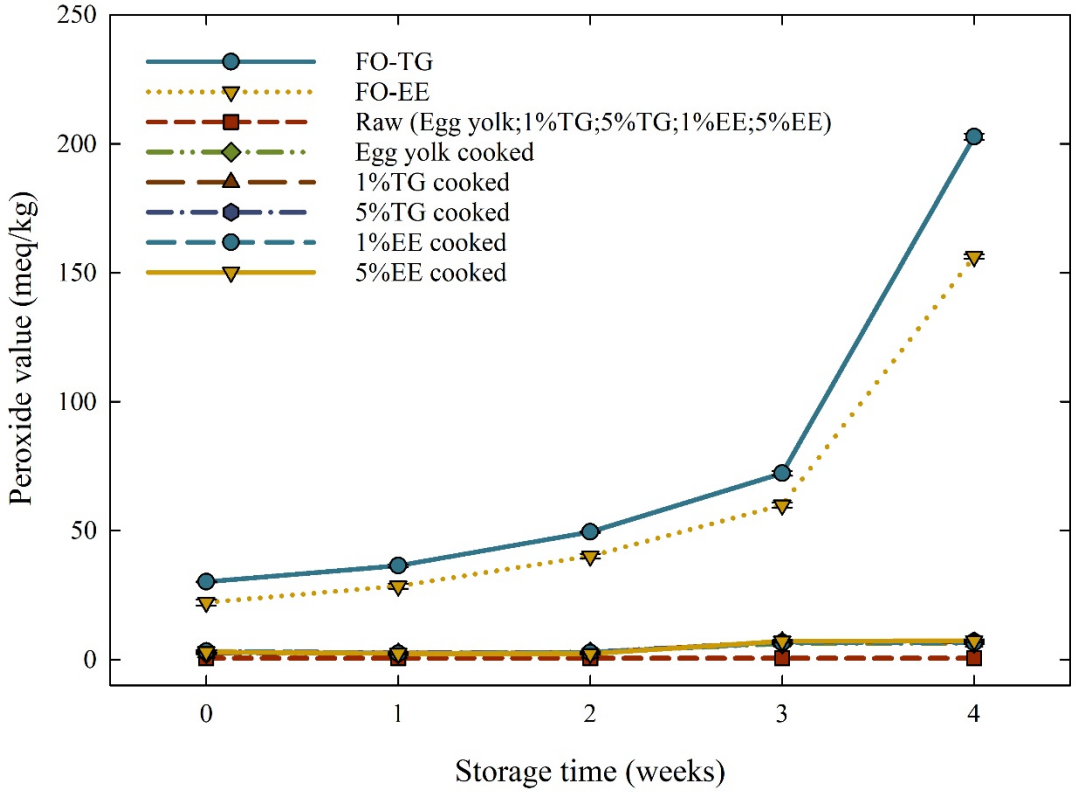

Figure 3.7 Peroxide value of fish oil triglycerides (FO-TG), fish oil ethyl esters (FO-EE), egg yolk and emulsions before (Raw) and after cooking (Cooked), over 4 weeks of storage at 4–6 °C. Raw samples were group after showing no significant differences at an α= 0.05. Each point corresponds to the mean ± standard error of three replications.

The POV was also assessed in emulsions subjected to cooking conditions (frying). Cooked samples from emulsions stored less than 3 weeks showed a POV ranging from 2.3–3.4 meq/kg, after subtracting the POV of the blank (canola oil). After the third week of storage, there was a significant increase in the POV of the cooked samples; however, this increment was far below the values displayed by the controls FO-TG and FO-EE. The POV of egg yolk and emulsions after cooking was not significantly different. This result indicates that the peroxides are being formed from the oxidation of lipids in the surface of the cooked samples.

3.4.4.2 Propanal content

Propanal has been widely used as a specific marker for oxidation of *n*-3 LC-PUFAs (Frankel et al., 2002; Chung et al., 2010). FO-TG and FO-EE processed at week 0 had 42.6±0.8 and 30.2±1.0 μg propanal/g, respectively. This propanal content was mainly attributed to the lack of protection from the processing conditions, which in this case included long times at moderately high temperatures (60 °C).

The propanal formation in fish oil triglycerides and ethyl esters followed a similar trend during the first three weeks; and at the fourth week it increased by 76 and 59%, for FO-TG and FO-EE respectively, in comparison with week 3 (Figure 3.8). The steep increase observed in the POV and propanal content on week 4 suggests that the peroxides and propanal formed at week 3 might be acting as prooxidants.

It has been shown that smaller particle size is not necessarily ideal to retard oxidation in lipid-containing microcapsules because of the larger surface area, which can result in higher surface oil concentration (Anwar and Kunz, 2011). However, in this study, no propanal was detected in the egg yolk and the emulsions at week 0 or during storage, thus proving that the native particle size distribution of the egg yolk results in high emulsion stability and prevents from oxidation up to four weeks under refrigeration conditions. No propanal formation was detected in cooked samples during the first two weeks of storage. After the third week, a small peak of propanal was detected, giving a concentration of <0.5 μg propanal/g sample. This value indicates the beginning of *n*-3 LC-PUFAs oxidation; nevertheless, this oxidation seems to be delayed by the protection offered by the egg yolk.

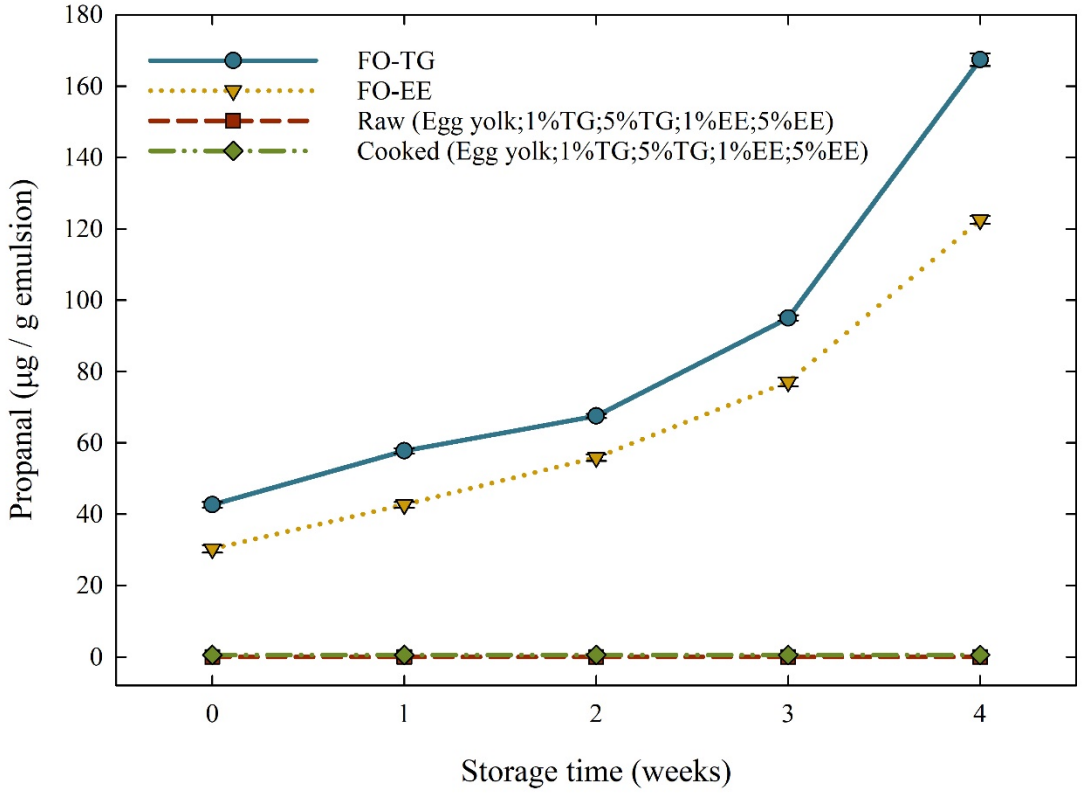

Figure 3.8 Propanal formation in fish oil triglycerides (FO-TG), fish oil ethyl esters (FO-EE), egg yolk and emulsions before (Raw) and after cooking (Cooked), over 4 weeks of storage at 4-6 °C. Raw and cooked samples were group after showing no significant differences at an α= 0.05. Each point corresponds to the mean ± standard error of three replications.

2.5 Conclusions

Egg yolk emulsion showed a bimodal particle size distribution and was affected by the type of fish oil added. Fish oil triglycerides, but not the ethyl esters, increased the average particle size of the egg yolk, in particular the micro-fraction. The high EEf achieved in all the treatments tested in this study, along with the negligible change in the surface oil concentration over time, showed the ability of egg yolk as a whole food matrix to form a stable emulsion with fish oil. In addition, oxidation tests proved the high efficiency of egg yolk to protect EPA+DHA from oxidation. Studying the bioavailability of the encapsulated EPA+DHA after gastrointestinal digestion would provide a better understanding of the potential of egg yolk to be used as their delivery system. Nevertheless, the results obtained from this work are especially significant since

using whole egg yolk is easier, simpler and more cost-effective to encapsulate EPA+DHA than using cost wall materials (such as casein) that were mostly reported in literature. As an excellent emulsifier, there is no need to add other emulsifiers or surfactants to stabilize the emulsions

CHAPTER 4: Effect of phospholipase A_1 and high-pressure homogenization on the functional properties, toxicity, and permeability of egg yolk/fish oil emulsions

4.1 Abstract

Enzymatic treatment of egg yolk with phospholipases can enhance its emulsifying properties and thermal stability. Additionally, a two-step process (primary and secondary homogenization) could form emulsions with better stability. Thus, the objective of this study was to assess the effect of enzymatic treatment and processing on the stability of egg yolk / fish oil emulsions. Egg yolk solutions (30% solids) before and after phospholipase A_1 treatment were used as carriers of different concentrations of fish oil (17, 29, 38, 44, and 50%, w/w). Emulsions were formed by primary (24000 rpm, 4 min) and/or secondary (200 MPa) homogenization and stored over 10 days at 45 °C. The effects of treatment and time on the particle size and distribution, viscosity, encapsulation efficiency, oxidative stability, cytotoxicity, and permeability of the emulsions was assessed according to a split-split-plot in time design. A significant increase in egg yolk apparent viscosity was observed after treatment with PLA_1. Emulsions formed by secondary homogenization displayed a more homogenous particle size distribution and had lower or not significant changes in particle size over time, while those formed by primary homogenization showed signs of aggregation after 4 days of storage. Secondary homogenization also increased the encapsulation efficiency of the carriers and reduced the release of oil to the particle surface. Samples processed with secondary homogenization showed, overall, a lower content of oxidation products. None of the emulsions were toxic to Caco-2 cells over the storage period; however, non-encapsulated fish oil stored for 10 days, reduced cell viability to 81%, at a concentration of 75 μg/mL medium. Only eicosapentaenoic acid was detected in the basolateral side of Caco-2:HT29 monolayers. The apparent permeability of EPA from non-encapsulated fish oil was significantly lower than that from encapsulated emulsions. Overall, the combined treatment of phospholipase A_1 and secondary homogenization resulted in emulsions with improved stability for up to 10 days at 45 °C.

4.2 Introduction

Egg yolk is used in a wide variety of food products, especially those that are emulsion-based, making it an important ingredient in the food industry. Numerous studies have been performed to further improve the applications and enhancement of egg yolk nutritional and functional properties. For instance, egg yolk is of great interest for the development of functional food products, due to the possibility of adding *n*-3 fatty acids through simple feed strategy (Arantes et al., 2009; Coorey et al., 2015; Lemahieu et al., 2015). Enhancing the content of long-chain omega-3 polyunsaturated fatty acids (*n*-3 LC-PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in egg yolk has been extensively explored due to their associated benefits to human health. These fatty acids are considered essential, since the human body cannot synthetize them. Although most studies have been focused on enhancing the content of these nutrients by modifying the hen's feed, the content and type of omega-3 fatty acids are greatly affected by the type of feed, age of bird, and many other environmental factors. Furthermore, long-term feeding with high-fat diet was reported to lower hen's productivity due to a laxative effect (Coorey et al., 2015). Additionally, hen is inefficient in biotransformation of alpha-linolenic acid into EPA and DHA as the later can only be obtained from marine source. Therefore, other approaches have been studied to produce egg products with higher content of *n*-3 fatty acids without the feeding approach drawbacks.

In the first chapter of this thesis, we demonstrated that shelf-stable egg yolk products with higher content of *n*-3 LC-PUFAs can be developed via processing. The use of egg yolk to encapsulate 1 and 5% of fish oil with high content of EPA and DHA resulted in 100% encapsulation efficiency. Furthermore, the products were stable for up to 4 weeks under refrigeration conditions, with no significant EPA and DHA degradation due to oxidation. The resulting products from our first study were developed to be used directly by the final consumers. However, preliminary tests showed that the main disadvantage of this approach was the consumer acceptance of the products tested (egg yolk omelets). Therefore, further studies were needed to improve the scope of applications of the developed egg yolk emulsions; for example, to be used as a functional food ingredient. Our approach was to increase the concentrations of EPA+DHA in the emulsions, with the potential advantage of using less wall material, or carrier, that could help reduce its cost. On the other hand, using higher EPA+DHA concentrations could result in less stable emulsions (Choi et al., 2010).

One way to address this task is by enhancing the egg yolk's functional properties by enzymatic modification. Enzymatic treatment of egg yolk with phospholipases has been shown to enhance its emulsifying properties and thermal stability (Dutilh and Growe, 1981; Buxmann et al., 2010; Jin et al., 2013; Strixner et al., 2013). Thermal stability is also a desired characteristic for food ingredients, due to more potential applications in food processing. Another consideration during the development of emulsion-based delivery systems is the processing technology employed. In order to obtain more stable emulsions, a two-step process is usually carried out. These steps can be classified as primary and secondary homogenization. In primary homogenization, the emulsion is formed directly by mixing two separated liquids, usually by high shear mixing, and results in a coarse emulsion with large droplet size. The role of secondary homogenization is to reduce the droplet size that can result in higher stability of the emulsions by minimizing coalescence (McClements, 2016).

We hypothesized that a combination of enzymatic treatment and processing technology can improve the functional properties of egg yolk, resulting in better stability when incorporating higher concentrations of EPA+DHA, characteristics desired for an efficient carrier of *n*-3 LC-PUFAs. Therefore, the objective of this research was to assess the effect of treatment with phospholipase A_1 and primary and secondary homogenization on the physicochemical properties of egg yolk emulsions with the addition of fish oil. Moreover, the toxicity of the emulsions and non-encapsulated fish oil stored under accelerated shelf-life conditions was tested in Caco-2 cells. The permeability of EPA and DHA from fish oil and emulsions in Caco-2: HT29 monolayers was also assessed.

4.3 Materials and methods

4.3.1 Materials

Fresh white shell eggs were collected from the Alberta Poultry Research Centre (Edmonton, AB, Canada) and used within 3 days of storage at 4–6 °C. Egg yolks were separated manually from the whites and carefully rolled on Whatman no.1 paper to eliminate albumen residues. The vitelline membrane was then punctured with a spatula and the egg yolk content was collected in a beaker placed in an ice bath and used immediately.

Fish oil from Alaska Pollock (*Gadus chalcogrammus*), containing ≥82% EPA+DHA as triglycerides (TG), was provided by AlaskOmega Products (Coshocton, Ohio, USA). Dulbecco's

modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate buffer saline (PBS), Hank's balanced salt solution (HBSS), 0.25% (w/v) trypsin–0.53 mM EDTA, 1% non-essential amino acids, 1% (w/v) penicillin–streptomycin, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Gibco Invitrogen (Burlington, ON, Canada). HT29-MTX-E12 cells, phospholipase A_1 (PLA_1, expressed in *Aspergillus oryzae*), *in vitro* toxicology assay kit MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) based, and all reagents used for chemical analyses were obtained from Sigma-Aldrich (Oakville, ON, Canada). Caco-2 cells were obtained from the American Type Culture Collection.

4.3.2 Egg yolk pre-treatment

4.3.2.1 Egg yolk solutions preparation

Three aliquots of freshly extracted egg yolk batch were taken to determine moisture content. The analysis was conducted using a convection oven set a 105–110 °C for 5 h to achieve constant weight. Each egg yolk batch was adjusted to 30% solids with distilled water. This step was conducted to facilitate the enzymatic reaction and was applied to the raw egg yolk samples to make accurate comparisons during the chemical analyses.

4.3.2.2 Enzymatic treatment using phospholipase A_1

The egg yolk solution was placed in a jacketed beaker and let settle at a constant temperature of 50 °C with gentle mixing. The initial pH of the solution was measured and adjusted to 6±0.01, followed by addition of 0.05% (w/w on egg yolk dry matter basis) of PLA_1 (activity ≥ 10,000 U/g; density 1.2 g/mL). The reaction was allowed for 5 h keeping the pH constant at 6±0.01 by adding 0.1M NaOH solution using a Titrando (Metrohm, Ionenstrasse, Switzerland). The enzymatic reaction was stopped by heating the samples at 72 °C for 5 min. PLA_1-treated egg yolk samples were kept at 4–6 °C and used within 24 hours for emulsion preparation.

4.3.3 Sample identification

Emulsions were formed by a combination of carrier, processing method, and concentrations of fish oil. Sample IDs were structured as follows: *RS17*. The first letter refers to the carrier type, R for raw egg yolk or P for phospholipase A_1-treated egg yolk. The second letter indicates the process used for the emulsion formation; S means primary (simple) homogenization and H

secondary (high-pressure) homogenization. Finally, the following two digits refer to the percentage of fish oil in the final emulsion on dry matter basis (17, 29, 38, 44, and 50%, w/w). Each group of treatments was paired with its control carrier without fish oil. For instance, Control-RS and Control-RH refers to raw egg yolk processed by primary and secondary homogenization, respectively. The same logic was applied for controls using PLA_1-treated egg yolk (Control-PS and Control-PH). Non-encapsulated fish oil triglycerides (FO) was used as negative control for toxicity assays.

4.3.4 Emulsion formation

The encapsulation of fish oil, regardless the carrier, was conducted by two different methods: primary and secondary homogenization, as described below.

4.3.4.1 Primary (simple) homogenization

Fish oil was added to the corresponding carrier and mixed manually to facilitate the dispersion of the oil. Emulsions were then formed by stirring the mixture at 24,000 rpm for 4 min using a T25 Ultra Turrax (IKA Works Inc., Wilmington, USA).

4.3.4.2 Secondary (high-pressure) homogenization

Emulsions prepared by secondary homogenization were first manually mixed (carrier + fish oil) then pre-homogenized at 14,000 rpm for 1 min, followed by high-pressure homogenization in a Pressure Cell Homogenizer FPG 12800 (Stansted Fluid Power LTD, London, UK) at 200 MPa and a hydraulic relief pressure of 240 MPa.

The emulsions were stored in individual air-tight sterile plastic tubes over 10 days at 45 °C, to represent accelerated shelf-life storage, and analyzed at days 0, 2, 4, 6, 8, and 10. Treatments with all five levels of concentration of fish oil and the positive controls were characterized according to their particle size and distribution. However, only emulsions containing 50% fish oil (regardless the processing conditions) were analyzed for viscosity, encapsulation efficiency, oxidative stability, cytotoxicity, and permeability.

4.3.5 Viscosity

The apparent viscosity, at 25 °C, of samples containing 50% fish oil and the controls were analyzed by steady state shear measurements (0.1–100 s^{-1}) in a Modular Compact Rheometer 302 (Anton Paar, Graz, Austria) equipped with a 50 mm diameter parallel plate measuring tool, using a 1 mm gap.

4.3.6 Particle size and distribution (PSD)

All emulsions and the controls (-PS and -PH) were analyzed for PSD at day 0 (freshly prepared) and after 2, 4, 6, 8, and 10 days of storage at 45 °C. Measurements were conducted at the same time on each testing day. Samples were diluted 1:4 (w/v) with a 1% (w/w) sodium dodecyl sulfate solution, then vortexed for 30 s prior to analysis to avoid multiple scattering. 2 mL of the diluted samples were placed in disposable polystyrene cuvettes for measurement. The particle size (as given by the Sauter mean diameter, d_{32}) and distribution of the emulsions were measured by dynamic light scattering (DLS) in a Litesizer™ 500 (Anton Paar, Graz, Austria) using backscattered angle (175 degrees), at 25 °C, and 2 min of equilibration time.

4.3.7 Encapsulation efficiency (EEf)

The encapsulation efficiency of the emulsions was determined by quantifying EPA+DHA in the total and surface oil, using the following formula:

$$EEf\ (\%) = [(\text{Total EPA+DHA} - \text{Surface EPA+DHA})/\text{Total EPA+DHA}] \times 100 \quad (1)$$

4.3.7.1 Total oil

Total oil was extracted using the method proposed by Bligh and Dyer (1959) with some modifications, as described in Chapter 2, section 3.3.6.1.

4.3.7.2 Surface oil

Surface oil in the emulsions was extracted by mixing 2 g samples with 10 mL hexane and vortexed for 40 s. This process was repeated twice. The hexane fractions were mixed then filtered using syringe filters with a membrane pore size of 0.45 μm; the solvent was collected in a glass tube and evaporated under nitrogen stream until constant weight.

Total and surface oil samples were kept in air-tight glass tubes capped with nitrogen and stored at -20 °C for no more than 12 hours before methylation.

4.3.7.3 Methylation and EPA+DHA quantification

The methylation and quantification of EPA+DHA was conducted following the method developed by Joseph and Ackman (1992) with some modifications. Due to the high concentration of EPA+DHA in the emulsions, 10 mg (±0.1 mg) oil sample were mixed with 1 mg of tricosanoic acid (23:0) methyl esters as internal standard. On the other hand, the internal standard was reduced to 0.1 mg to obtain clearer EPA+DHA peaks in samples from permeability tests. The area counts of EPA and DHA were obtained by mass chromatography to quantify EPA+DHA triglycerides, following the methodology and formulas described in Chapter 2, section 2.3.6.3.

Total EPA+DHA naturally present in egg yolk (controls) was also quantified and deducted from the emulsions when applicable. Surface and total EPA+DHA were also measured on day 10 to determine the amount of EPA+DHA that remained encapsulated after storage at 45 °C.

4.3.8 Propanal content

The propanal formation in FO and emulsions containing 50% fish oil was monitored over 10 days at 45 °C by headspace chromatography. The methodology used has been previously described in Chapter 1, section 3.8. Propanal content was calculated using a standard curve (r^2= 0.99) built with 0, 3, 6, 9, 12, and 15 µg propanal (98% purity) in Milli-Q water and expressed as µg propanal/g sample.

4.3.9 Peroxide value (POV)

The quantification of peroxides in FO and emulsions containing 50% fish oil over 10 days at 45 °C was conducted. The POV was measured following the AOAC official method (965.33), described in Chapter 2, section 2.3.7. The peroxide value was calculated using the following formula:

meq peroxide/kg oil= (mL $Na_2S_2O_3$ × molarity $Na_2S_2O_3$ × 1000)/g test portion (3)

4.3.10 Toxicity of emulsions in Caco-2 cells

Caco-2 cells from passages 19–25 were used for toxicology study. Cells were routinely grown in supplemented DMEM, refreshing the medium every other day. At 80% confluence, cells were harvested using PBS containing 0.25% trypsin and 0.53 mM EDTA and used for seeding.

The cytotoxicity of emulsions containing 50% fish oil was evaluated at 0, 2, 4, 6, 8, and 10 days of storage at 45 °C. Controls without fish oil were also tested to exclude the influence of the carrier. Non-encapsulated fish oil (FO) was used as negative control to compare the influence of using egg yolk as carrier. The toxicity of the emulsions was determined by measuring viability of Caco-2 cells, following the *In Vitro* Toxicology assay from Sigma-Aldrich, based on the MTT method developed by Mosmann (1983).

Caco-2 cells were seeded in high-glucose and l-glutamine DMEM medium supplemented with 10% FBS, 1% non-essential amino acids, 1% penicillin and streptomycin, and 2.5% (v/v) HEPES to a concentration of 50,000 cell/mL. Then, 10000 cells / well (200 μL) were placed on a 96-well plate and incubated at 37 °C in a humidified incubator with an air-CO_2 (95:5, v/v) atmosphere for 24 h. After incubation, the original medium was replaced with the corresponding emulsion or FO suspended in supplemented DMEM to final concentrations of 50, 75, and 100 μg fish oil/mL medium, and incubated for 24 h. This analysis was conducted for day 0 samples and used to screen for the best concentration of fish oil to test the influence of storage time in the emulsions as well as permeability in monolayers (section 2.7). These concentrations accounted for 41, 62, and 82 μg EPA+DHA/mL medium. After the second incubation, the wells were gently washed 3 times with PBS. The cells were then incubated for 4 h with DMEM medium containing 10% MTT solution (5 mg/mL PBS). The medium was then removed, and the formazan crystals formed on each well were dissolved pipetting up and down with 200 μL of acidified isopropanol. The absorbance of each well was measured spectrophotometrically at a wavelength of 570 nm. The background absorbance of the plate was measured at 690 nm and subtracted from the value at 570nm. Supplemented DMEM without emulsions was used as blank reference for 100% viability. This final result was used to calculate relative cell viability (%) using the following formula:

$$\text{Relative cell viability (\%)} = (\text{Abs treated cells} * 100)/(\text{Abs blank}) \quad (4)$$

4.3.11 Permeability assay in Caco-2:HT29 membranes

The permeability of encapsulated and non-encapsulated fish oil was tested on intestinal epithelial membranes. Caco-2 cells were used at passages 24–32. HT29-MTX-E12 cells were used at passages 30–45. Both cell lines were routinely grown in supplemented DMEM, refreshing the medium every other day. At 80% confluence, cells were harvested using PBS containing 0.25% trypsin and 0.53mM EDTA and used for seeding. Freshly prepared emulsions containing 50% fish oil and fish oil without any processing were used in this test.

Caco2 and HT29 cells at a ratio of 8:2 and a density of 2.5 x 10^5 cells/mL were seeded on Corning trans-well plates (pore size 0.4 μm, surface area 1.12 cm^2). The cells were incubated at 37 °C in a humidified incubator with an air-CO2 (95:5, v/v) atmosphere for 21 days, refreshing the medium every other day on the first and second weeks, then every day on the third week. The integrity of the cell monolayers was determined by measuring the trans-epithelial electrical resistance (TEER) using an epithelial tissue Volt/Ohm meter (EVOM2, World Precision Instruments, Saratosa, FL, USA). Monolayers presenting TEER values of 750 Ω / cm^2 and higher (Akbari et al., 2017) were selected for the tests. On the 22nd day of incubation, cell medium was replaced with sterile HBSS (buffered with 30 mM HEPES) at pH 7.4 and incubated at 37 °C for 30 min. The HBSS in the apical side of the trans-wells was removed and 0.5 mL HBSS containing emulsion or non-encapsulated fish oil for a total of 75 μg fish oil/mL emulsion. The permeability test was conducted at 37 °C for 9 h, sampling the basolateral side at 3, 6 and 9 h; the sample taken was replaced to keep 1.5 mL total HBSS in the basolateral side. TEER values were also measured at the end of the incubation to ensure values above 400 Ω / cm^2. Fatty acids in the basolateral side were extracted by mixing the samples with isooctane at a ratio of 1:1.5 (sample: isooctane), heated to 35–40 °C then vortexed at for 30 s while still warm. The mix was let stand until complete clarification then the isooctane layer was separated; this process was repeated twice. The isooctane extracts were combined, mixed and dried under nitrogen stream and proceed to quantify EPA+DHA triglycerides or ethyl esters as described in section 3.3.7.3. The apparent permeability coefficient (P_{app}) was calculated using the following equation:

$$P_{app} = dQ / dt \times 1 / AC_0 \quad (5)$$

where dQ / dt is the permeability rate (the slope of EPA+DHA concentration graph as function of time); A is the surface area of the membrane filter (cm^2); and C_0 is the initial concentration of EPA+DHA in the apical side (mg/mL).

4.3.12 Statistical analysis

This study was a BACI (Before-After-Control-Impact) experiment, aimed to evaluate the changes in responses of a given treatment over time. A Split-split-plot in time experimental design was used. The main plots were each batch of samples using a specific carrier. The fixed effects applied to the experimental units were 2 levels of processing, 6 levels of fish oil concentration (including level 0 as control), and 6 levels of time. All chemical analyses were conducted three times. Four to six replications were performed for biological analysis. Results were expressed as the mean and the standard error. A one-way analysis of variance (ANOVA) was used to find significant factors between treatments at one given time. Repeated measures ANOVA were used to find significant factors over time. Significant differences of the mean values were considered at $p < 0.05$ using Tukey test. For toxicity tests, an adjusted p value was used to determine significant differences between samples and the negative control. All the statistics were conducted using the statistical package R version 3.3.2 in R studio version 1.0.136 (R Core Team, 2016).

4.4 Results and discussion

4.4.1 Statistical significance and interactions among the factors

The Analysis of Variance, set at an $\alpha= 0.05$, showed that the processing type and concentration of fish oil had a significant influence on the characteristics of the emulsions using raw or PLA_1-treated egg yolk as carriers. Additionally, there were significant differences between main plots at a specific level of treatment, showing the influence of carrier type on the characteristics of the emulsions. The repeated measurements analysis of variance showed that storage time was also a significant factor influencing the toxicity of the negative control (FO). Moreover, the interaction of treatment*time also had a significant influence on the stability and toxicity of the samples.

4.4.2 Treatment-dependent viscosity

The apparent viscosity of the samples changed depending on the carrier and the processing type. All samples, regardless of the treatment, showed a shear-thinning behavior at increasing shear rate (Figure 4.1). Figure 4.1a shows that emulsions using PLA_1-treated egg yolk (PS50) as carrier had a significantly higher apparent viscosity compared to those using raw egg yolk (RS50). This effect was also observed after treating diluted egg yolk (70:30, w/w) with

phospholipase D from *Streptomyces chromofuscus*. Buxmann et al. (2010) found that the increased viscosity was dependent on the incubation time and was positively correlated with the formation of phosphatidic acid. The increased viscosity was attributed to structural changes of the spherical LDL micelles due to alterations of the interactions between apoproteins and phospholipids at the particle surface. A similar mechanism effect has been observed by the action of PLA_1 on egg yolk that could also explain the increased viscosity observed in our study. PLA_1 cleavages the ester bond at the sn-1 position of the triglyceride and converts phospholipids into lysophospholipids. This action results in the alteration of the native spherical structure of the LDL micelles, which causes destabilization of the micelles and promotes interactions between apoproteins and phospholipids at the surface of the particle (Kumar and Mahadevan, 1970; Jin et al., 2013). Emulsions containing 50% fish oil, regardless of the carrier, showed an increased apparent viscosity at shear rates of 0.1–10 1/s after secondary homogenization (Figure 4.1b). However, the viscosity followed a similar pattern at shear rates higher than 10.

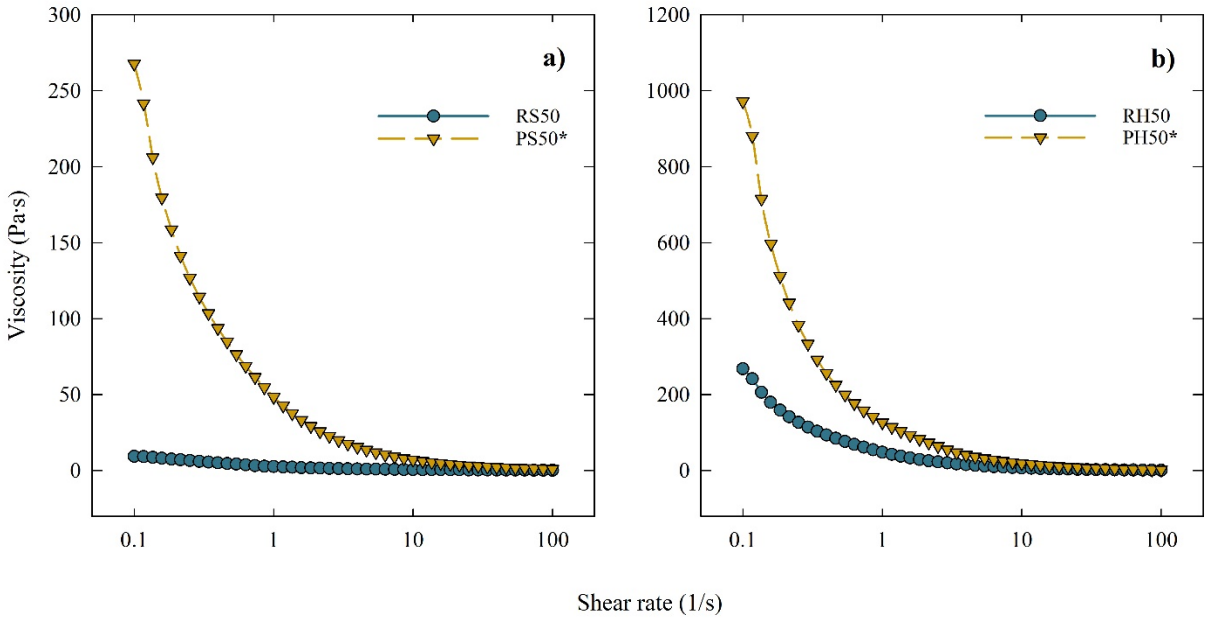

Figure 4.1 Apparent viscosity of emulsions containing 50% fish oil (w/w egg yolk dry matter) as affected by processing. a) using raw or PLA_1-treated egg yolk after primary (RS50 and PS50, respectively), and b) secondary homogenization (RH50 and PH50). * indicates significantly higher than the other treatment at the same homogenization conditions.

These observations can be attributed to the partial unfolding of egg yolk proteins that have undergone shear stress caused during high-pressure homogenization. During the formation of an emulsion, proteins that are adsorbed at the oil/water interface experience structural changes that might affect the rheological properties of the emulsion. Lee et al. (2007) observed the loss of β-sheet structures of whey proteins after high-pressure homogenization at 50 MPa; this effect was similar to that caused by thermal treatment at 70 °C. It has also been reported that hydrostatic high-pressure treatment of 500 MPa for 10 min caused significant denaturation of duck's egg yolk proteins decreasing its solubility to 84.3% (Lai et al., 2010).

The increased viscosity could also be associated with the breakage of fat globules (egg yolk liposomes + fish oil) caused by high turbulent and shear stress during high-pressure homogenization, which caused re-arrangement of the microstructure of the egg yolk, as further discussed in section 4.4.3. Additionally, a partial decrease of the viscosity of the egg yolk/fish oil emulsions was observed at higher concentrations of fish oil in the emulsions, regardless the processing and carrier type (*data not shown*). This behavior could be related to the reported decreased viscosity of egg yolk right after the first viscosity maximum is achieved in temperature-dependent viscosity curves, which was attributed to the release of neutral lipids from the core of LDL micelles (Werlein, 1987). The same effect was observed by Chalamaiah et al. (2018), where higher lipid content in egg yolk resulted in a decreased viscosity compared to its granule fraction after phosvitin depletion.

4.4.3 Effect of treatment on the PSD of emulsions

In this study, effects of primary and secondary homogenization on the particle size and distribution of the emulsions were studied. For primary homogenization, egg yolk was directly mixed with fish oil using a high shear mixer at 24,000 rpm, while secondary homogenization was conducted in a high-pressure valve homogenizer at 200 MPa.

Changes in the particle size and distribution of the emulsions over accelerated shelf-life conditions were measured by dynamic light scattering (DLS). In DLS, the particle size is given by the light scattered by the random motion of particles suspended in a liquid is detected and recorded many times. DLS considers that smaller particles move faster than larger ones.

The analysis of variance showed a significant influence (α= 0.05) of the carrier, processing type, and concentration of fish oil on the particle size and distribution of the

emulsions. The influence of storage time on the average particle size of emulsions using raw or PLA_1-treated egg yolk as carriers are shown in Figures 4.2 and 4.3, respectively.

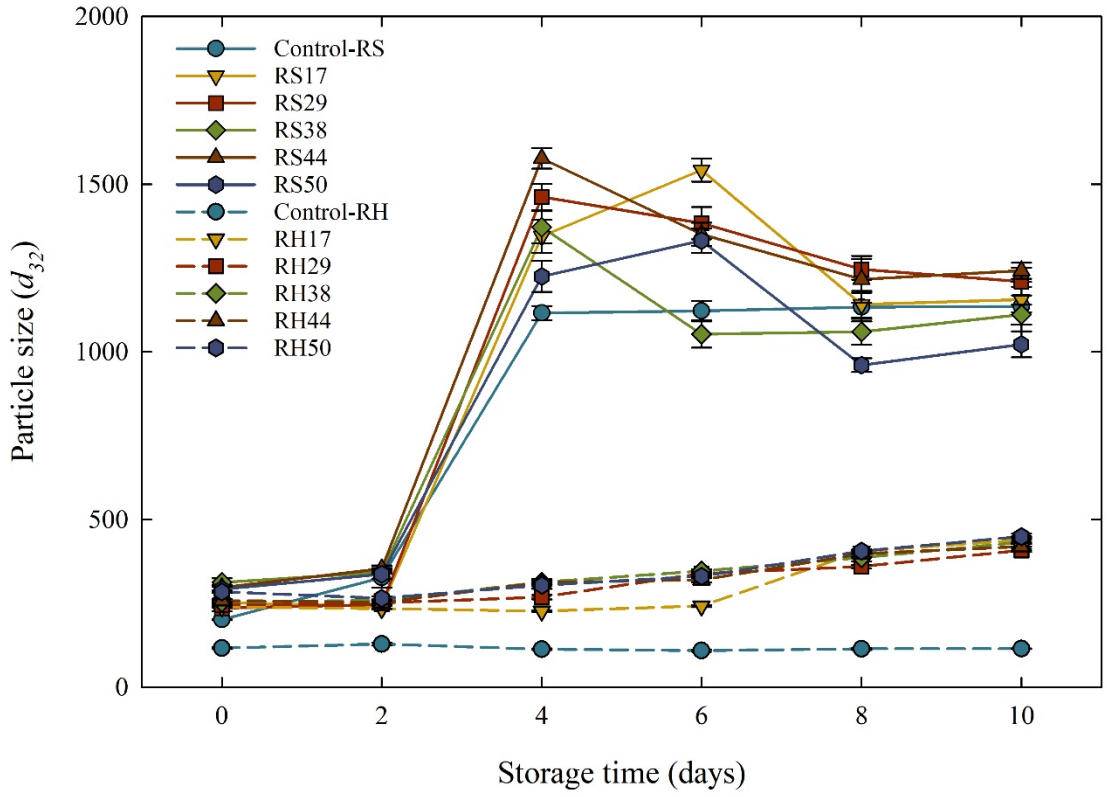

Figure 4.2 Average particle size of emulsions formed by simple (S) and high-pressure homogenization (H), using raw egg yolk (R) as carrier over 10 days of storage at 45 °C. The last two digits of each code refer to the concentration of fish on dry matter basis. Each data point corresponds to the mean ± the standard error of three replications.

In a high-pressure valve homogenizer, samples undergo intense turbulence which is characterized by chaotic fluctuations in the velocity of the fluid with time and location. The extremely large shear and pressure gradients generated during turbulence causes disruption of droplets (McClements, 2016), resulting in a smaller particle size. Figure 4.2 shows that the initial particle size of the raw egg yolk control after secondary homogenization (Control-RH) was significantly smaller ($p < 0.05$) than the control after primary homogenization (Control-RS). Qian and McClements (2011) also reported a significant decrease in the average particle size of β-lactoglobulin: corn oil emulsions after high-pressure homogenization ranging from 4 to 14 kbar. Most emulsions using raw egg yolk as carrier, regardless the processing type, had similar

particle size at day 0; yet the size of emulsions prepared by secondary homogenization remained without significant changes over time. On the other hand, emulsions formed by primary homogenization displayed a significant increase of the average particle size at day 4 of storage. This behavior could be partially attributed to the aggregation of the particles caused by polymerization of the egg yolk proteins (Salminem et al., 2014).

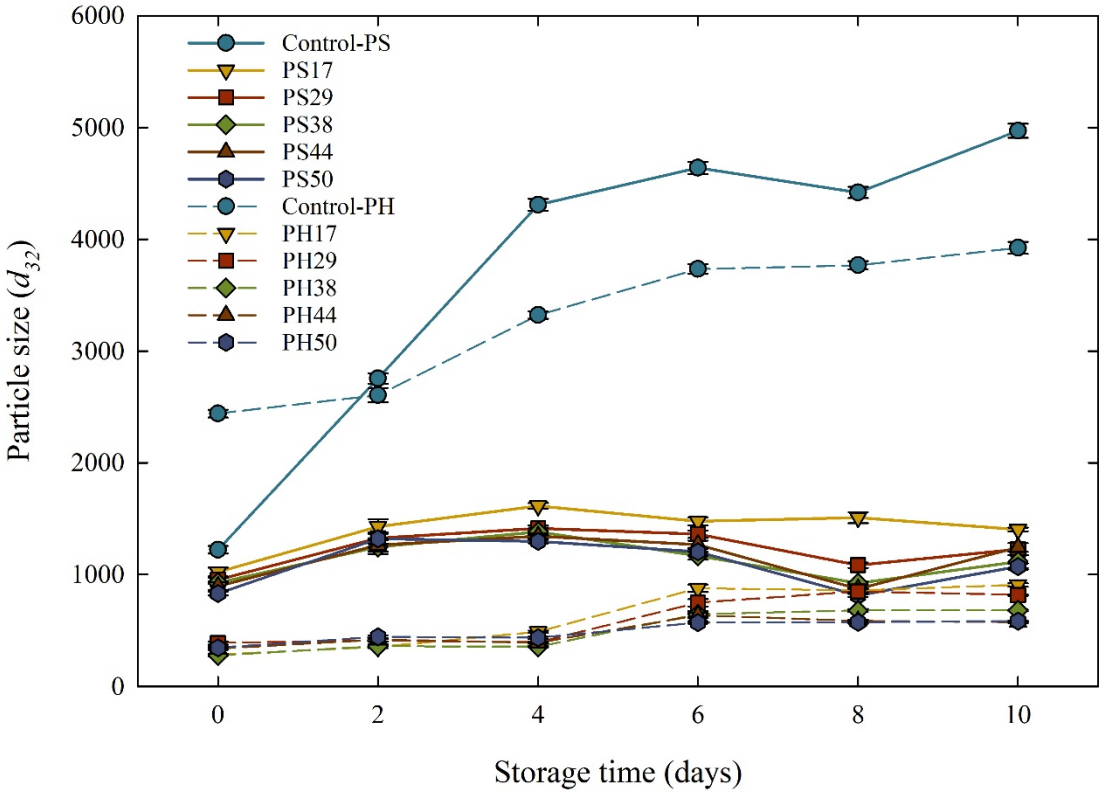

Figure 4.3 Average particle size of emulsions formed by simple (S) and high-pressure homogenization (H) of phospholipase A_1-treated egg yolk (P) as carrier over 10 days of storage at 45 °C. The last two digits of each code refer to the concentration of fish on dry matter basis. Each data point corresponds to the mean ± standard error of three replications.

For emulsions using PLA_1-treated egg yolk as carrier, the concentration of fish oil and the interaction of fish oil*processing type had higher influence than time ($p < 0.01$). Figure 4.3 shows that at higher concentrations of fish oil, the average particle size was smaller and showed more stability over time, as observed for samples containing 44 and 50% fish oil (PH44 and PH50, respectively). The opposite behavior was observed in controls without fish oil (Control-PS

and Control-PH), for which the highest increase in particle size was obtained at day 10. Overall, samples using PLA_1-treated egg yolk as carrier, and their corresponding control, had larger particle size than those using raw egg yolk. It has been reported that treatment with PLA_1 changes the native structure of egg yolk granules, dissociating phosphocalcic bridges and releasing fatty acids (Jin et al., 2013). These changes could result in particles with larger surface area that are reflected on a higher average particle size.

Figure 4.4a illustrates the particle size distribution of emulsions formed by primary homogenization, using raw or PLA_1-treated egg yolk (RS50 and PS50, respectively). A wider distribution was observed for PS50, showing peaks at 10 nm, 50 nm, and 3 μm; while RS50 mean peak shows at 20 nm and a smaller density at 400 nm. After high-pressure homogenization, the main peaks of both samples appeared in the same range (20-60 nm), while the 3 μm fraction observed in PS50 completely disappeared in PH50 (Figure 4.4b). A similar effect was observed by Floury et al. (2000). The authors reported a higher proportion of particles in the nano-range and smaller proportion around 10 μm of whey proteins: sunflower oil emulsions homogenized at 150 MPa compared to those at 20MPa.

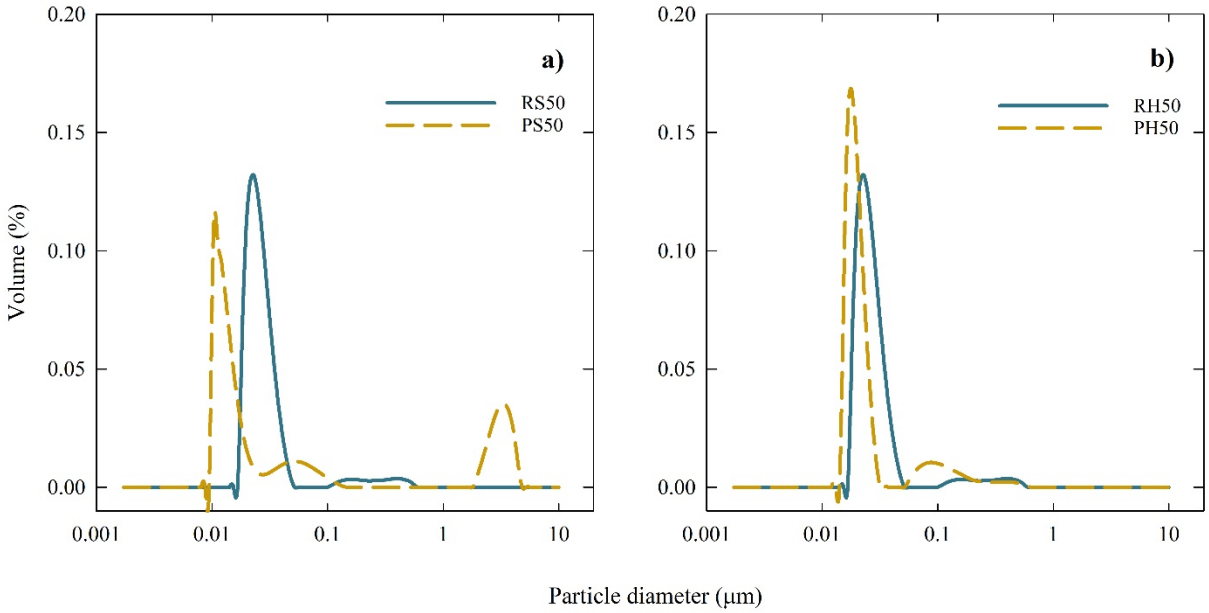

Figure 4.4 Particle distribution of emulsions containing 50% fish oil (w/w egg yolk dry matter) as affected by processing. a) using raw or PLA_1-treated egg yolk after primary (RS50 and PS50, respectively), and b) secondary homogenization (RH50 and PH50).

4.4.4 Influence of treatment on the EPA+DHA encapsulation efficiency of egg yolk

The encapsulation efficiency was assessed in freshly prepared emulsions (Day 0), and after 10 days of storage at 45 °C; the total encapsulated oil was quantified and the relative percentage (compared to total encapsulated oil at day 0) was reported. These results are summarized in Table 4.1. The Repeated Measures ANOVA showed a significant influence of treatment, time, and their interaction on the encapsulation efficiency of the emulsions. High-pressure homogenization significantly increased the encapsulation efficiency of the carriers and stability of the emulsions over time, as shown by the lower decrease in the total encapsulated oil at day 10, in comparison to the lost in emulsions formed by simple homogenization. It has been widely reported that enzymatic modification of egg yolk with phospholipases increases its heat stability and its emulsifying capacity (Buxmann et al., 2010; Jin et al., 2013; Strixner et al., 2013). In this study we observed the same effect on the PLA_1-treated emulsions. PLA_1-treated egg yolk emulsions resulted in not only a higher EEf at day 0 but also in improved stability over storage, as shown by the higher percentage of fish oil that remained encapsulated after 10 days of storage. Although all emulsions had significant loss of encapsulated oil over time, the combination of enzymatic treatment and high-pressure homogenization resulted in the most efficient and stable emulsion. On the other hand, sample RS50 was the most affected by time, displaying the greatest loss of encapsulated oil.

Table 4.1 Encapsulation efficiency of emulsions after primary and secondary homogenization and remaining encapsulated oil after 10 days of storage at 45 °C. Treatments correspond to emulsions containing 50% (w/w) fish oil after primary and secondary homogenization using raw (RS50 and RH50) or PLA_1-treated (PS50 and PH50) egg yolk. Every value corresponds to the mean + standard error of three replications. Different letters within columns mean significant differences among treatments. The encapsulated oil was significantly lower for all treatments at day 10. Significance was considered at $p < 0.05$.

Treatment	Encapsulation Efficiency (%) at Day 0	Encapsulated oil (%) at Day 10
RS50	97.4 ± 0.01^{b}	81.9 ± 0.90^{c}
RH50	99.9 ± 0.01^{a}	94.3 ± 0.76^{ab}
PS50	98.9 ± 0.01^{ab}	92.7 ± 0.30^{b}
PH50	99.9 ± 0.27^{a}	95.9 ± 0.05^{a}

4.4.5 Oxidative stability of emulsions under accelerated shelf-life conditions

In order to assess the oxidative stability of samples under accelerated shelf-life conditions, the propanal content and peroxide value were measured. These analyses were conducted in non-encapsulated fish oil (FO) and emulsions with the highest content of fish oil (50% dry matter basis) at 0, 2, 4, 6, 8, and 10 days of storage at 45 °C.

Samples processed with high-pressure homogenization showed, overall, a lower content of oxidation products. Both POV and propanal showed the same trend as seen in Figure 4.5. The highest increase in peroxide value and propanal was observed from day 4 to day 8, with a slight decrease on day 10.

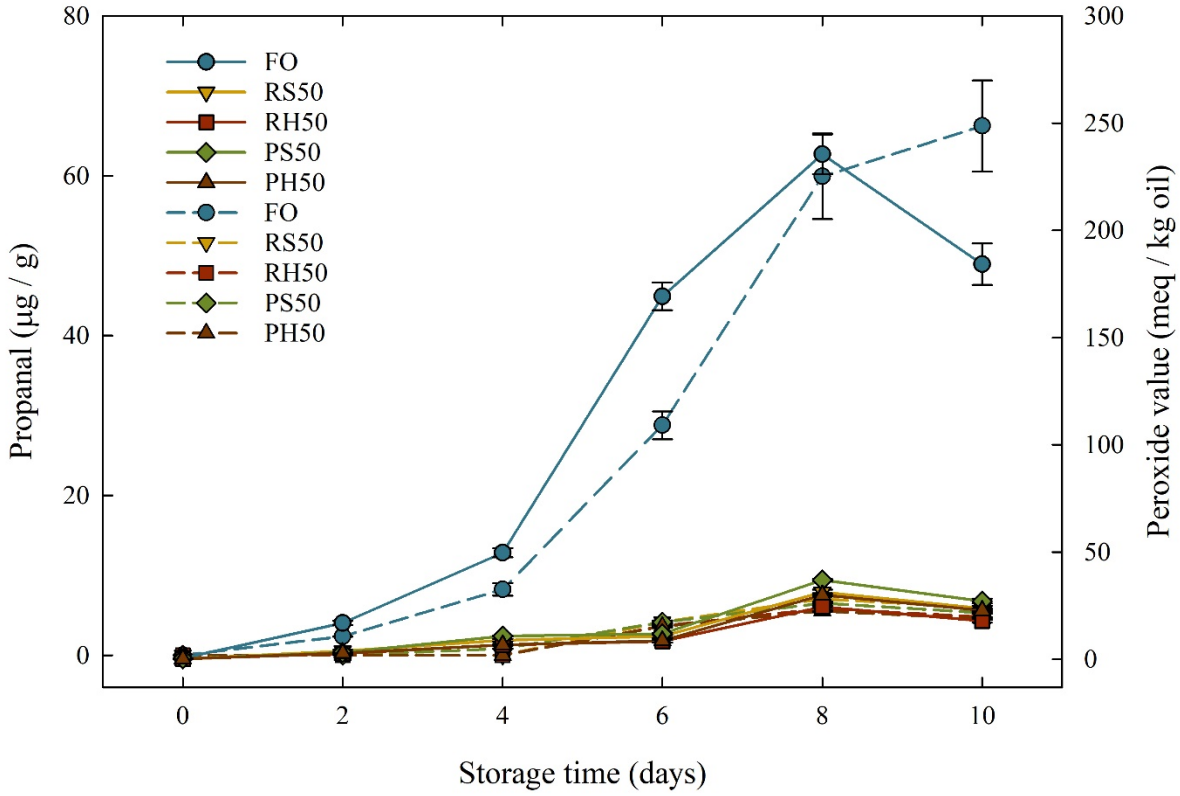

Figure 4.5 Oxidative stability of the emulsions containing 50% (w/w egg yolk dry mater basis) and non-encapsulated fish oil triglycerides (FO), after primary (S) and secondary (H) homogenization over 10 days of storage at 45 °C, as given by the propanal content and the peroxide value. Peroxide values are represented by solid lines while propanal content is represented by dotted lines. Each data point represents the mean ± standard error of three replications.

Unlike peroxide formation, propanal was not detected in emulsions at day 0 and day 2. A significant reduction of peroxides and secondary oxidation products of fish oil was reported in a study using triestearin, quillaja saponins, and lecithin as emulsifiers. The effect was partially attributed to the tannins and polyphenols naturally present in the quillaja extracts (Salminem et al., 2014).

Although after day 8 of storage there was significant increase in the POV and propanal content in the emulsions, the values were significantly lower (6 and 26-fold on average, respectively) than those previously found for non-encapsulated fish oil (*data not shown*). Egg yolk is also known for its components with antioxidant activity, such as carotenes. These natural antioxidants could also function as an extra aid in the prevention of oxidation of *n*-3 LC-PUFAs.

4.4.6 Toxicity of the emulsions under accelerated shelf-life conditions

The MTT test used to assess the toxicity of the emulsions measures the activity of mitochondrial dehydrogenases, which cleave the tetrazolium ring, forming purple formazan crystals. Once the crystals are dissolved, the intensity of the purple solution determines the dehydrogenases activity and thus, cell viability. The results were expressed as relative cell viability percentage, considering the absorbance of cells incubated with supplemented DMEM without emulsion (blank) as 100%. A value < 85% of the blank absorbance was considered as toxic (Gilman and Cashman, 2007). To exclude a non-specific effect of the carriers on the cells, the controls were tested simultaneously with the treatments. The controls showed no significant effect on the viability of Caco-2 cells under accelerated shelf-life conditions (*data not shown*); therefore, the observed effects were attributed to the oxidation of fish oil.

Figure 4.6 shows the relative cell viability of three concentrations for each emulsion and the negative control at day 0. None of the treatments showed significant differences with those cells incubated with supplemented DMEM. However, lower variability was observed in samples containing 75 μg fish oil/mL emulsion; therefore, this concentration was used for toxicity analyses over time.

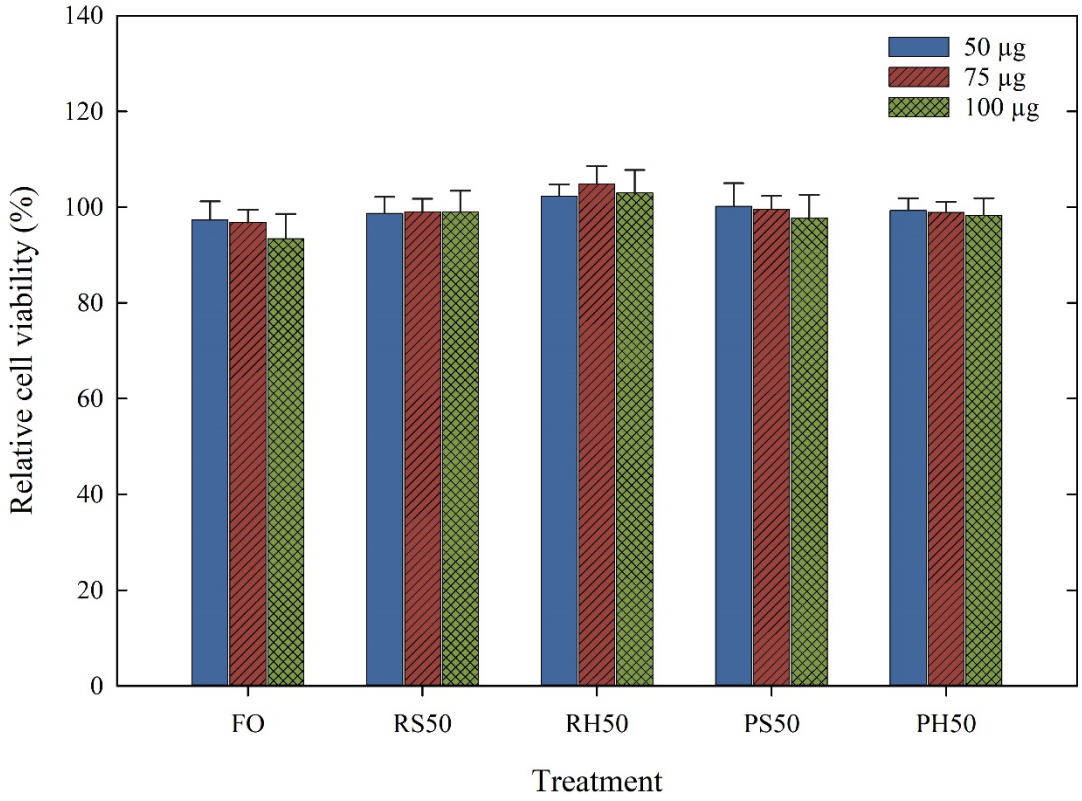

Figure 4.6 Toxicity of emulsions freshly prepared containing 50% (w/w egg yolk dry mater basis) fish oil, using raw (R) or PLA_1-treated egg yolk (P), after primary (S) or secondary (H) homogenization, and non-encapsulated fish oil triglycerides subjected to secondary homogenization (FO). Each bar represents the mean ± standard error of four to six replications of each treatment tested at 3 different concentrations (µg fish oil/mL medium) on day 0. No significant differences with the blank (medium without emulsions) were found at α= 0.05.

The effect of storage on the toxicity of emulsions and the negative control is shown in Figure 4.7. During the first 6 days of storage, viability was not affected. At day 8, FO showed an average cell viability of 74.5% compared to the average blank. However, the statistical analysis showed that this was only significantly lower (p= 0.0462) than 95% of viability of the blank. Considering that a component is toxic when the viability of cells is reduced under 85%, FO showed toxicity at day 10 of storage at 45 °C, when its average cell viability (71.08%) was significantly lower (p= 0.0384) than 81% of the cell viability of the blank.

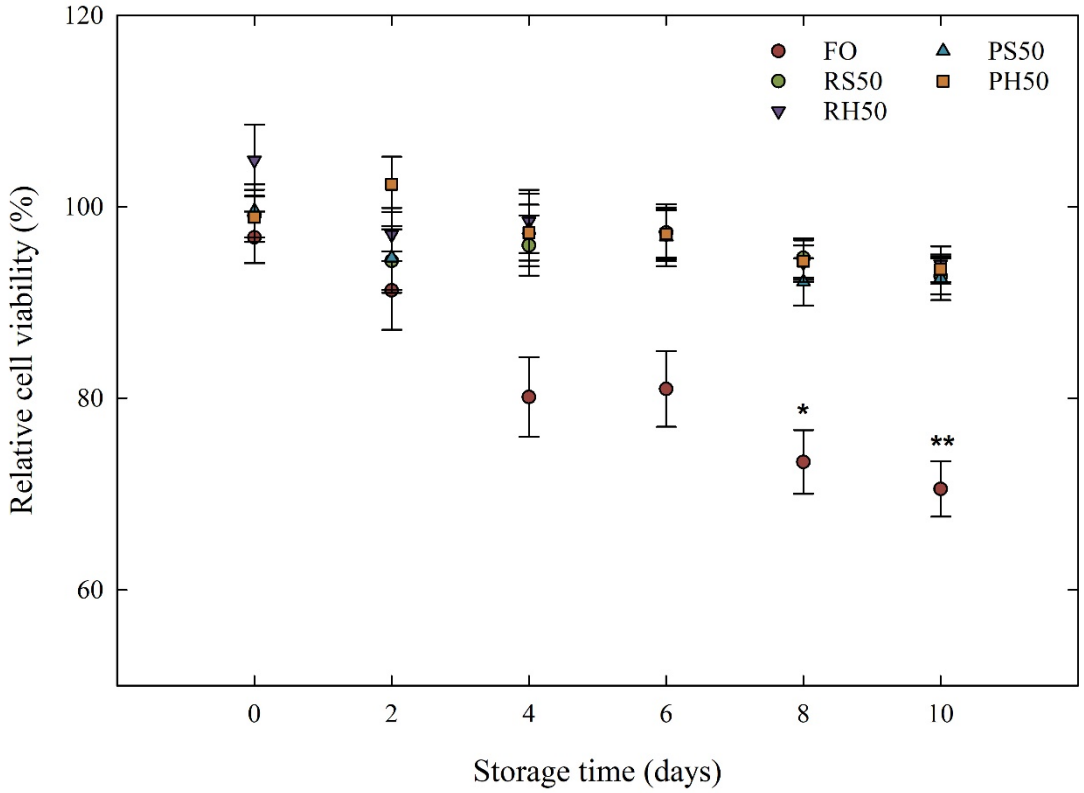

Figure 4.7 Toxicity of the emulsions containing 50% (w/w egg yolk dry mater basis), using raw (R) or PLA_1-treated egg yolk (P), after primary (S) or secondary (H) homogenization, and non-encapsulated fish oil triglycerides subjected to secondary homogenization (FO), over 10 days of storage at 45 °C. All samples were tested at a concentration of 75 μg fish oil/mL medium. Each point represents the mean ± standard error of four to six replications. Toxicity after 8 and 10 days of storage is indicated by * and ** (significantly lower than 85% and 80% relative cell viability, respectively).

A time-dependent decrease in Caco-2 cells proliferation has also been reported when testing EPA and DHA from fish oil using glycerol as emulsifier (Jordan and Stein, 2003). The study reported the half-maximal inhibitory concentration (IC_{50}) for EPA and DHA of 45 and 34 μM, respectively, after 72 h of incubation; nevertheless, the control used to exclude a non-specific lipid effect (soybean oil + glycerol) showed a decrease of about 30% under the same conditions. These observations are in agreement with our results, since the non-encapsulated fish oil showed higher reductions in cell viability over time. Moreover, another study using EPA and DHA from fish oil (Gilman and Cashman, 2007), found a decrease of Caco-2 cells viability ~ 85% when using 80 μM EPA + 80 μM DHA (50 μg/mL medium). In another study, it was shown that lutein emulsions containing Tween 20 (20% v/v) as emulsifier were cytotoxic at

concentrations as low as of 14 μg/mL medium. The toxicity was proven to be due to the carrier and not the lutein itself. On the other hand, emulsions using β-lactoglobulin as an emulsifier, did not show toxic effect at a concentration of 420 μg/mL (Frede et al., 2014). In comparison, our results also showed that raw egg yolk and phospholipase A1-treated egg yolk had no toxic effect on Caco-2 cells, thus highlighting the importance of using food-grade materials to encapsulate lipophilic compounds.

4.4.7 Apparent permeability of encapsulated and non-encapsulated EPA+DHA

The apparent permeability coefficient was used to assess the ability of encapsulated and non-encapsulated EPA+DHA to pass through the intestinal epithelial cell monolayer barrier of the gastrointestinal tract. Caco-2 cells differentiated to resemble the small intestinal enterocytes, along with the presence of mucus secreting cells (HT29) derived from globet cells, were used to form the monolayers.

Caco-2:HT29 monolayers were incubated with 75 μg fish oil/mL medium, accounting for a total of 46 μg EPA and 16 μg DHA in the apical side. The basolateral side of the trans-well plates was sampled at 3, 6, and 9 h of incubation. After analysis of fatty acids in the basolateral side, EPA was detected but no DHA. One of the factors affecting these results could be the higher susceptibility of DHA to oxidation (Lee et al., 2003). Oxidized DHA have been shown to affecting the transport proteins in Caco-2 cells, consequently reducing its uptake efficiency (Maestre et al., 2013; Dasilva et al., 2018). In an *in vitro* study, Dasilva et al. (2018) found that the uptake of DHA in Caco-2 cells is largely decreased after gastric oxidation; while EPA seems to be more stable under the same conditions. In addition, the authors also found an influence of the EPA:DHA ratio on the uptake efficiency of DHA. A 1:1 ratio of EPA:DHA showed the lowest decrease in the uptake of oxidized DHA. In our study, the EPA:DHA ratio was about 3:1; which could also have affected the transport of DHA.

Based on this observation, the P_{app} was calculated based on the EPA content in the apical and basolateral compartments. It has been previously shown that a smaller particle size increases the uptake of lutein-loaded particles (Frede et al., 2014). Figure 4.8 shows the Papp of four treatments and the negative control. It was found that the Papp of RS50 was significantly lower (α= 0.05) than those of RH50, PS50, and PH50, but higher than FO. Moreover, there was no significant difference between RH50, PS50, and PH50.

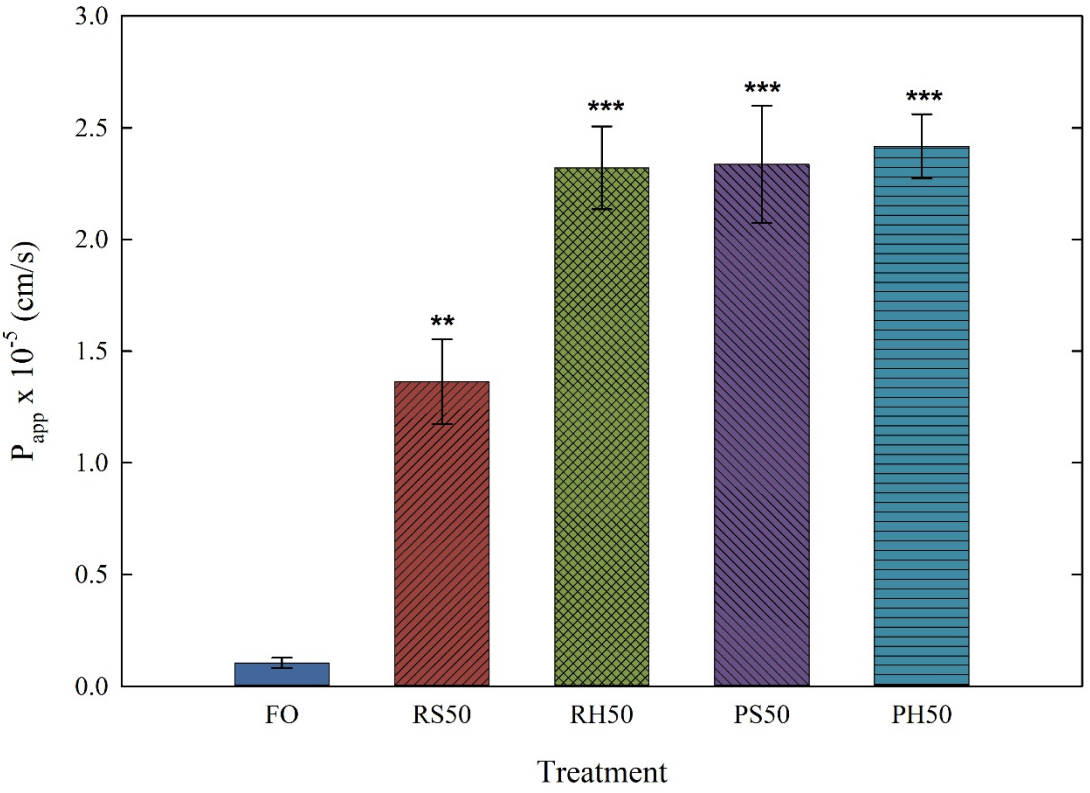

Figure 4.8 Apparent permeability of EPA from emulsions containing 50% (w/w egg yolk dry mater basis) fish oil, using raw (R) or PLA_1-treated egg yolk (P), after primary (S) or secondary (H) homogenization, and from non-encapsulated fish oil triglycerides subjected to secondary homogenization (FO), over 10 days of storage at 45 °C. All samples were tested at a concentration of 75 μg fish oil/mL medium. Levels of significance are shown by ** and *** indicating significantly greater at $p < 0.01$ and $p < 0.001$, respectively. Each bar represents the mean ± standard error of four replications.

Additionally, the basolateral samples of all the emulsions showed the presence of octanoic acid, ranging from concentrations of 0.25–0.35 (area%). It has been shown that HT29-CL19A cells express the lysophosphatidic receptor 2 as response to lysophosphatidic acid from food (Li et al., 2005). Moreover, differentiated Caco-2 cells are able to secrete enzymes with lysophospholipase and L-α-glycerophosphocholine-phosphodiesterase activity which are capable of converting lysophosphatidylcholine and monoacylglycerols ingested orally into free fatty acids (Inaba et al., 2014). The combined activities of these enzymes could be responsible for the production of medium-chain fatty acids, such as octanoic acid, from the metabolism of egg yolk, especially when modified with PLA_1. Furthermore, the sodium salt of octanoic acid has shown a

positive effect in drug absorption in Caco-2 cell monolayers (Lindmark et al., 1995). This effect could also be related to the higher permeability of EPA obtained in the emulsions where egg yolk was used as carrier rather than non-encapsulated fish oil. Further studies are needed to show the potential correlation between the production of octanoic acid and the transport of long-chain fatty acids in cell monolayers.

4.5 Conclusions

The combined treatment of phospholipase A_1 and high-pressure homogenization resulted in emulsions with improved stability for up to 10 days at 45 °C, in comparison with those using raw egg yolk and simple homogenization. The average particle size of samples RH50 and PH50 showed the highest stability over time; while the particle size of the controls significantly increased during storage. Additionally, egg yolk emulsions decreased the oxidation rate of EPA+DHA as shown by the lower propanal and peroxides formation that resulted in significantly lower toxicity in Caco-2 cells compared to that of non-encapsulated fish oil. This effect was related to the low formation of oxidation products. Furthermore, the permeability of EPA through Caco-2:HT29 membranes was significantly improved when high-pressure homogenized emulsions were used as carriers, compared to non-encapsulated fish oil. The results obtained in this work are of special significance showing promising applications of a whole food matrix, egg yolk, as delivery system of *n*-3 LC-PUFAs in food products.

CHAPTER 5: Stability of egg yolk / polysaccharide emulsions encapsulating EPA+DHA under accelerated shelf-life conditions

5.1 Abstract

Polysaccharides can form interfacial complexes with proteins to form emulsions with enhanced stability. In this chapter we assessed the effect of two polysaccharides, two fish oil esterification types, and two processing methods on the stability of egg yolk/fish oil emulsions, following a split-split-plot in time design. Egg yolk/fish oil mixtures were added with a polysaccharide solution to a final concentration of 0.25 g/100 g of emulsion, then subjected to primary (24,000 rpm, 4 min) and/or secondary (200 MPa) homogenization. Emulsions were stored at 45 °C for up to 10 days and characterized according to their particle size and distribution, viscosity, encapsulation efficiency, oxidative stability, and cytotoxicity. Emulsions containing triglycerides had a higher viscosity than those containing ethyl esters. Emulsions containing gum guar had higher viscosity than those containing gum arabic. The size distribution of egg yolk/gum guar emulsions were narrower and skewed to the smaller size end after secondary homogenization. The mean particle size obtained for emulsions containing triglycerides were significantly larger than those with ethyl esters. Emulsions containing gum arabic were the most affected by time and displayed a bridging flocculation effect. Despite this bridging effect, the core material remained encapsulated within the continuous phase of the emulsion, as shown by the similar encapsulation efficiency compared to treatments using gum guar. However, gum arabic treatments showed higher release of oil to the particle surface over time. Additionally, secondary homogenization aided to reduce the accumulation of surface oil. The stability of egg yolk/polysaccharide emulsions was given mostly by the ability of the polysaccharide to act as an extra barrier to entrap the lipids in the core material and not due to the particle size of the emulsion. Surface oil in emulsions after 10 days of storage was related to the production of oxidation compounds such as peroxides and propanal. No significant toxicity in Caco-2 cells viability was found from emulsions over time. However, non-encapsulated fish oil, regardless of esterification type, showed a decrease of about 80% viability after 10 days of storage. These results support the potential of egg yolk/polysaccharide systems to form emulsions with better stability.

5.2 Introduction

Emulsions show great potential for use as delivery systems for lipophilic bioactive compounds, mainly for applications in food and pharmaceutical products. The effectiveness of these delivery systems is assessed by the degree of protection they offer to the encapsulated compounds, or core material, from environmental stresses like those encountered during food processing and later in the gastrointestinal tract. Emulsions are most commonly prepared using a biopolymer that acts as an emulsifier to form a single layer surrounding the core material (Ramakrishnan et al, 2013; Frede et al., 2014; Bai et al., 2016). However, producing double-layer emulsion systems has been gaining attraction in the scientific community due to their potentially enhanced properties when compared with conventional monolayer emulsions (Chung et al., 2014; Chivero et al., 2015; Jimenez-Martin et al., 2015; Chang et al., 2016; Xu et al., 2017). An emulsion's stability is dependent on multiple factors, all of which need to be taken into consideration: 1. As droplets are undergoing constant movement, they eventually collide with each other, leading to destabilization of the system. 2. Chemical reactions occurring in the droplet core, such as oxidation of lipids, can also cause destabilization (McClements, 2016). 3. The selection of biopolymers for use as emulsifiers in an emulsion system will greatly influence its stability over time. Thus, the type and molecular structure, and the charge of such biopolymers are important features that should be considered as they determine the functionality of the emulsion (Qin et al, 2016). 4. an emulsifier can also generate steric or electrostatic interactions that prevents droplets from aggregation (Chanamai and McClements, 2002; Xu et al., 2017).

The primary coating in a double-layer emulsion is usually achieved with proteins, whereas the secondary layer can be formed using polysaccharides (Chung et al., 2014; Chang et al., 2016). Polysaccharides can form interfacial complexes with the proteins used in monolayer emulsions to coat the oil droplets; these complexes reduce the van der Waals attractions between proteins and increase the steric and electrostatic repulsion between droplets (Güzey and McClements, 2006).

Additionally, polysaccharides have been extensively used in the food industry as thickening and gelling agents; they have also shown to be resistant to pH changes, ionic strength, and harsh temperatures such as heating and freezing (Dickinson, 2008; Güzey and McClements, 2006; Xu et al., 2017). Therefore, there is great interest in studying their potential use as an extra barrier during the encapsulation of lipophilic bioactive compounds that are sensitive to such

conditions (Charoen et al., 2011). For instance, gum arabic, an anionic polysaccharide derived from the exudate of the Acacia Senegal tree, has been extensively used as an emulsifier for food applications. The emulsifying property of gum arabic is conferred by its amphiphilic nature: its hydrophobic side, the polypeptide chain, anchors the molecules to the lipid phase; while the arabinogalactan fraction (hydrophilic side) extends into the aqueous phase. The low viscosity of gum arabic makes it a highly employed emulsifier especially in beverages. On the other hand, guar gum forms solutions with high viscosity and pseudoplastic behavior. Guar gum is a linear nonionic polysaccharide which consists of a chain of β(1–4)-linked mannopyranosyl units with an α(1-6)-linked D-galactopyranosyl chain every second residue (Belitz and Grosch, 1999; McClements, 2016) and it is commonly used in the food industry as a thickening agent. The predominant stabilizing mechanism of polysaccharides is known to be steric, which, unlike proteins that generate electrostatic forces, has shown to form emulsions with better stability under different environmental stresses.

Furthermore, another important consideration is the processing technology selected to form the emulsion. For instance, high-pressure homogenization has been widely used to form mono and double-layer emulsions. The high shear force generated during the homogenization process entails the rapid movement of the encapsulating materials to the interfacial region and causes violent disruption of droplets, making most droplets in the nano size range < 200 nm (Wooster et al., 2008).

In Chapter 3 we showed that egg yolk, as a whole food matrix, can form stable emulsions with improved stability of fish oil under refrigeration conditions. However, significant losses of the encapsulated EPA+DHA were observed under high temperature conditions for extended storage, as reported in Chapter 4. As described above, emulsions stabilized by polysaccharides can be more resistant to temperature stresses; therefore, we hypothesized that the inclusion of a polysaccharide layer in egg yolk: fish oil emulsions can produce emulsions with better stability. Therefore, the objective of this study was to assess the effect of gum guar or gum arabic on the stability of egg yolk/fish oil emulsions. The performance of two processing conditions, simple and high-pressure homogenization was also evaluated.

5.3 Materials and methods

5.3.1 Materials

Egg yolks were manually separated (Chapter 3, Section 3.3.1) from fresh white shell eggs collected from the Alberta Poultry Research Centre (Edmonton, AB, Canada). Fish oil from Alaska Pollock (*Gadus chalcogrammus*) with high content of eicosapentaenoic (EPA) and docosahexaenoic (DHA) fatty acids as triglycerides or ethyl esters was obtained from AlaskOmega Products (Organic Technologies, Ohio, USA). Caco-2 cells were obtained from the American Type Culture Collection and used from passages 19–32. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate buffer saline (PBS), Hank's balanced salt solution (HBSS), 0.25% (w/v) trypsin–0.53 mM EDTA, 1% non-essential amino acids, 1% (w/v) penicillin – streptomycin, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Gibco Invitrogen (Burlington, ON, Canada). Gum guar, gum arabic, *In vitro* toxicology assay, and all reagents used for chemical analyses were obtained from Sigma-Aldrich (Oakville, ON, Canada).

5.3.2 Emulsion formation

The moisture content of each batch of freshly extracted egg yolk was determined using a convection oven set a 105–110 °C for 5 h; this information was collected to calculate the final polysaccharide solution: egg yolk: water ratio aiming for emulsions with a final concentration of 30% egg yolk solids. Polysaccharide solutions (1% w/w in distilled water) were prepared by stirring gum guar or gum arabic in cool Milli-Q water at 400 rpm until complete dissolution. The concentration and type of fish oil added followed the experimental design further described in section 5.3.5. Egg yolk/fish oil/polysaccharide emulsions were formed using two processing approaches: primary and secondary homogenization, as described below.

5.3.2.1 Primary (simple) homogenization

Fish oil was added to the egg yolk and mixed manually to facilitate its dispersion, followed by stirring at 24,000 rpm for 2 min using a T25 Ultra Turrax (IKA Works Inc., Wilmington, USA). The polysaccharide solution was then added to reach a final concentration of 0.25 g polysaccharide per 100 g of emulsion.

5.3.2.2 Secondary (high-pressure) homogenization

Fish oil: egg yolk mixtures were pre-mixed at 24,000 rpm for 2 min using a T25 Ultra Turrax. The corresponding polysaccharide solution was then added and mixed manually until homogeneous. This mixture was subjected secondary homogenization in a Pressure Cell Homogenizer FPG 12800 (Stansted Fluid Power LTD, London, UK) at 200 MPa and a hydraulic relief pressure of 240 MPa.

To simulate accelerated shelf-life conditions, freshly prepared samples were transferred to air-tight sterile plastic tubes and stored at 45 °C for up to 10 days; samples from the same experimental unit were taken at days 0 (freshly prepared samples), 2, 4, 6, 8 and 10 for further analysis. Based on preliminary results, all treatments were characterized according to their particle size and distribution over time. However, only selected treatments were analyzed for viscosity, encapsulation efficiency, oxidative stability, and cytotoxicity. Details about the sample groups are given on the results section.

5.3.3 Characterization of the emulsions

5.3.3.1 Viscosity

The apparent viscosity at 25 °C, was determined by steady state shear measurements (0.1–100 s^{-1}) in a Modular Compact Rheometer 302 (Anton Paar, Graz, Austria). A concentric cylinder measuring tool with an active length of 120.2 mm and 72.5 mm positioning length was used to determine viscosity curves of emulsions formed by primary homogenization. A 50 mm diameter parallel plate measuring tool and a 1 mm gap setting was used for emulsions resulting from secondary homogenization.

5.3.3.2 Particle size and distribution (PSD)

Samples were diluted 1:4 (w/w) with 1% (w/w) sodium dodecyl sulfate solution, then vortexed for 30 s prior to analysis to avoid multiple scattering. 2 mL samples were placed in a disposable polystyrene cuvette for measurement. The particle size (as given by the Sauter mean diameter, d_{32}) and distribution (given by the volume %) of the emulsions were measured by DLS in a Litesizer™ 500 (Anton Paar, Graz, Austria) using backscattered angle (175degrees), at 25 °C, and 2 min of equilibration time.

5.3.3.3 Encapsulation efficiency (EEf)

The encapsulation efficiency was determined by quantifying the total and surface EPA+DHA in the particles, using the following formula:

EEf (% = [(Total EPA+DHA – Surface EPA+DHA)/Total EPA+DHA] x 100 (1)

EPA+DHA in total and surface oil was extracted and quantified following the procedure described in detail in Chapter 3 section 3.3.6.1 and Chapter 4 section 4.3.7.2, respectively. In summary, total oil was extracted following the chloroform: methanol method adapted from Bligh and Dyer (1959) while surface oil was extracted by washing the samples with hexane. The methylation and quantification of EPA+DHA was conducted following the method developed by Joseph and Ackman (1992), using tricosanoic acid methyl esters or ethyl esters as internal standards for triglycerides or ethyl esters, respectively. Results were expressed as mg EPA or DHA/g oil.

5.3.3.4 Propanal content

The propanal formation in emulsions over 10 days of accelerated shelf-life conditions was monitored by headspace chromatography. About 2 g samples were placed into a 20 mL headspace vial, sealed and heated at 60 °C for 30 min, followed by 30 min of equilibration time at room temperature. The headspace was analyzed using a capillary gas chromatograph with a Restek Stabilwax (Crossbond Carbowax) column (Chapter 2, section 2.5.4). Propanal content was calculated using a standard curve (r^2= 0.99) built with 0, 3, 6, 9, 12, and 15 µg propanal (98% purity) in water and expressed in µg propanal/g emulsion.

5.3.3.5 Peroxide value

The peroxide value (POV) was measured following the AOAC official method 965.33, as described in section 2.5.5 of Chapter 2. POV was calculated using the following formula:

meq peroxide/kg oil= (mL $Na_2S_2O_3$ × molarity $Na_2S_2O_3$ × 1000)/g test portion (2)

5.3.4 Effect of emulsions on viability of Caco-2 cells

Caco-2 cells were routinely grown in supplemented DMEM, refreshing the medium every other day, and used for seeding at 80% confluence.

The toxicity of emulsions was evaluated at day 0, 2, 4, 6, 8, and 10 days of storage at 45 °C. Egg yolk/polysaccharide solutions without fish oil (controls) were tested at the same conditions to eliminate the effect of the carrier components. Non-encapsulated fish oil concentrates, FOT (triglycerides) and FOE (ethyl esters), were used as negative controls to highlight the effect of the carrier on the toxicity of fish oil.

The toxicity of the samples was assessed by measuring viability of Caco-2 cells, following the *In Vitro* Toxicology assay from Sigma-Aldrich, based on the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) method developed by Mosmann (1983).

Caco-2 cells were harvested with PBS containing 0.25% trypsin and 0.53 mM EDTA, then seeded in high-glucose and l-glutamine DMEM medium supplemented with 10% FBS, 1% non-essential amino acids, 1% penicillin and streptomycin, and 2.5% (v/v) HEPES to a concentration of 50,000 cells/mL. Then, 10,000 cells/well (200 µL) were placed on a 96-well plate and incubated at 37 °C in a humidified incubator with 5% CO_2 for 24 h.

After incubation, the original medium was replaced with the corresponding treatment suspended in the supplemented DMEM at a final concentration of 50, 75, and 100 µg EPA+DHA/mL medium and incubated for 24 h. For non-encapsulated fish oil (FOT and FOE), the same final concentration of EPA+DHA was added to the medium. Supplemented DMEM without emulsions was used as blank reference for 100% viability. After the second incubation, the wells were gently washed 3 times with PBS. The cells were then incubated for 4 h with DMEM medium containing 10% MTT solution (5 mg/mL PBS). The medium was then removed, and the formazan crystals formed on each well were dissolved pipetting up and down with 200 µL of acidified isopropanol. The absorbance of each well was measured spectrophotometrically at a wavelength of 570 nm. The background absorbance of the plate was measured at 690 nm and subtracted from the value at 570 nm. This final result was used to calculate relative cell viability (%) using the following formula:

$$\text{Relative cell viability (\%)} = (\text{Abs treated cells} * 100)/(\text{Abs blank}) \quad (3)$$

5.3.5 Sample identification and statistical analysis

Sample IDs were structured as follows: *A44TS*. The first letter refers to the polysaccharide type, A for gum arabic or *G* for gum guar. The following two digits refer to the carrier: EPA+DHA ratio expressed as percentage of triglycerides or ethyl esters on dry matter basis. The third

character indicates the esterification type, *T* for triglycerides and *E* for ethyl esters. Finally, the last letter refers to the process used for the emulsion formation; *S* means simple homogenization and *H* high-pressure homogenization. Each group of treatments was paired with its control without fish oil, identified as Control-AS, Control-AH, Control-GS, and Control-GH; where the last two letters mean as specified before. Non-encapsulated fish oil triglycerides or ethyl esters were used as negative controls for toxicity assays and oxidative stability.

Emulsions were formed using a combination of polysaccharide type, processing method, concentration and type of esterification of EPA+DHA. The experimental design was a Split-split-plot in time. The main plots were the egg yolk: polysaccharide solutions. The fixed factor for the split-plot was the processing type at two levels: simple or high-pressure homogenization. Finally, the fixed factor for the split-split-plot was the type and concentration of fish oil at 4 levels: 22 and 44% EPA+DHA triglycerides and 22 and 44% EPA+DHA ethyl esters. Time (at 6 levels) was considered a fixed effect since its effect on the output variables was of interest. All chemical analyses were carried out in triplicates. Four replications were conducted for the biological analysis. Results were expressed as the mean and the standard error. A linear mixed-effects model was used to assess the significance of time as a factor and the fixed factors over time; a one-way analysis of variance (ANOVA) was used to find significant factors among treatments at one given time. Significant differences of the mean values were considered at $p < 0.05$ using Tukey test. All the statistics were conducted using the statistical package R version 3.3.2 in R studio version 1.0.136 (R Core Team, 2016).

5.4 Results and discussion

A whole food matrix, egg yolk, was used as the primary emulsifier while gum arabic or guar were used to form a secondary layer. Simple and high-pressure homogenization were used as primary and secondary homogenization processes, respectively. The resulting emulsions were developed to protect lipophilic bioactive compounds, such as EPA and DHA, that are highly sensitive to environmental stresses. The effectiveness of the emulsions was given by their stability and their ability to prevent oxidation/degradation of EPA and DHA over 10 days of storage at 45 °C.

5.4.1 Statistical significance and interactions among the factors

A significance level of $\alpha = 0.05$ was used for all statistical analyses. The influence of the fixed factors on the physicochemical characteristics of the main plots (egg yolk/polysaccharide emulsions) was analyzed using the package "agricolae" in R. Differences between main plots over time were analyzed using a linear model. The Analysis of Variance showed a highly significant ($p < 0.001$) influence of treatment, and the interaction (treatment*time) on the average particle size, encapsulation efficiency, and toxicity of the emulsions. The type of polysaccharide used as secondary layer, showed a significant influence ($p < 0.01$) on the all the variables evaluated. On the other hand, time showed its highest influence ($p < 0.05$) only during interactions with factors. The effect of processing on the response variables was highly significant ($p < 0.001$). Finally, the highest influence ($p < 0.001$) of esterification type and concentration and their interaction with other factors was observed on the average particle size.

5.4.2 Treatment-dependent viscosity

It has been shown that the rheological properties of oil-in-water emulsions are mostly dependent on the original properties of the biopolymers used as wall materials and their effective volume occupied in the emulsion system (Ercelebi and Ibanoglu, 2009; Wu et al., 2009; Chung et al., 2014). In this study, a significant effect of the polysaccharide type, processing, and esterification of fish oil on the apparent viscosity of emulsions was observed. The effect of primary (24,000 rpm, 4 min) and secondary (200 MPa) homogenization on the viscosity curves of samples containing 44% EPA+DHA is displayed in Figure 5.1a and b.

Secondary homogenization had a highly significant influence ($p < 0.001$) on the apparent viscosity of all emulsions, regardless the esterification type. Nevertheless, the esterification type of the fatty acids in the fish oil had a significant influence ($p < 0.05$) on samples within the same processing type. For instance, samples containing triglycerides had a higher viscosity than those containing ethyl esters. Gum arabic is commonly used as emulsifier in beverage industries due to its low viscosity. On the other hand, gum guar is widely used in the food industry as thickening agent. It belongs to the galactomannan's family, which is known for its high water-binding capacity that allows them to form viscous emulsions even at low concentrations. Therefore, as expected, emulsions where gum guar was used as secondary layer had higher viscosity ($p < 0.05$) than those using gum arabic.

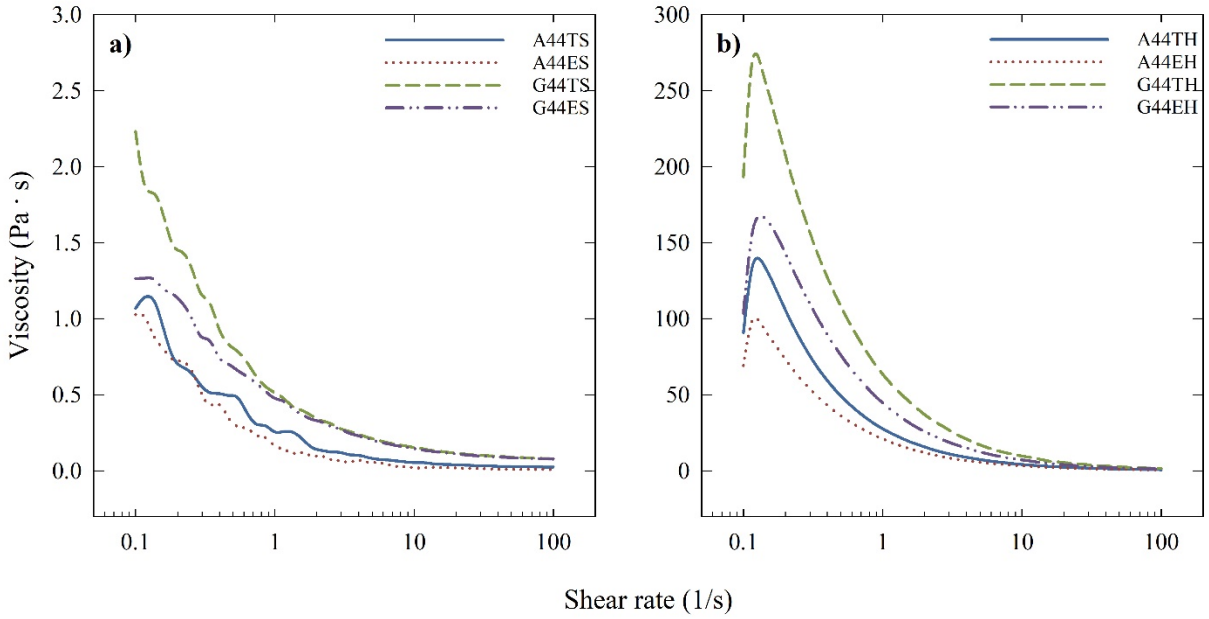

Figure 5.1 Effect of a) primary (S) and b) secondary (H) homogenization, on the apparent viscosity of egg yolk/fish oil triglycerides (T) or ethyl esters (E) emulsions containing gum arabic (A) or gum guar (G). Each curve is the average of three replications. The viscosity of all emulsions formed by secondary emulsification was significantly higher ($p < 0.001$) than those prepared by primary emulsification. G44TH was significantly more viscous ($p < 0.05$) than all other treatments under the same processing conditions.

5.4.3 Particle size and distribution as affected by treatment and time

5.4.3.1 Particle size distribution

Figures 5.2a and b show the effect of processing type on the particle size distribution of the emulsions containing 44% EPA+DHA triglycerides or ethyl esters. The distribution curves of samples using gum guar as the secondary layer were narrower and skewed to the smaller size end after secondary homogenization. This observation is in agreement with those reported by Bai et al (2016), who observed monomodal narrower particle distributions at higher homogenization pressures. Moreover, the distribution curves of emulsions containing gum arabic seemed to depend not only on the processing type but also on the esterification of the fish oil used as core material. Emulsions containing gum arabic and triglycerides processed by primary homogenization (A44TS) showed a second peak on the micro-size region that almost disappeared after secondary homogenization (A44TH). This peak could be the result of larger particles formed by the bridging flocculation effect (Xu et al, 2017). On the other hand, A44ES (gum arabic+ethyl esters after primary homogenization) did not show an obvious second peak

but the curve became more skewed to the lower size end after secondary homogenization. The overall changes in the distribution curves were also reflected in the average particle size of each treatment as discussed in the following section.

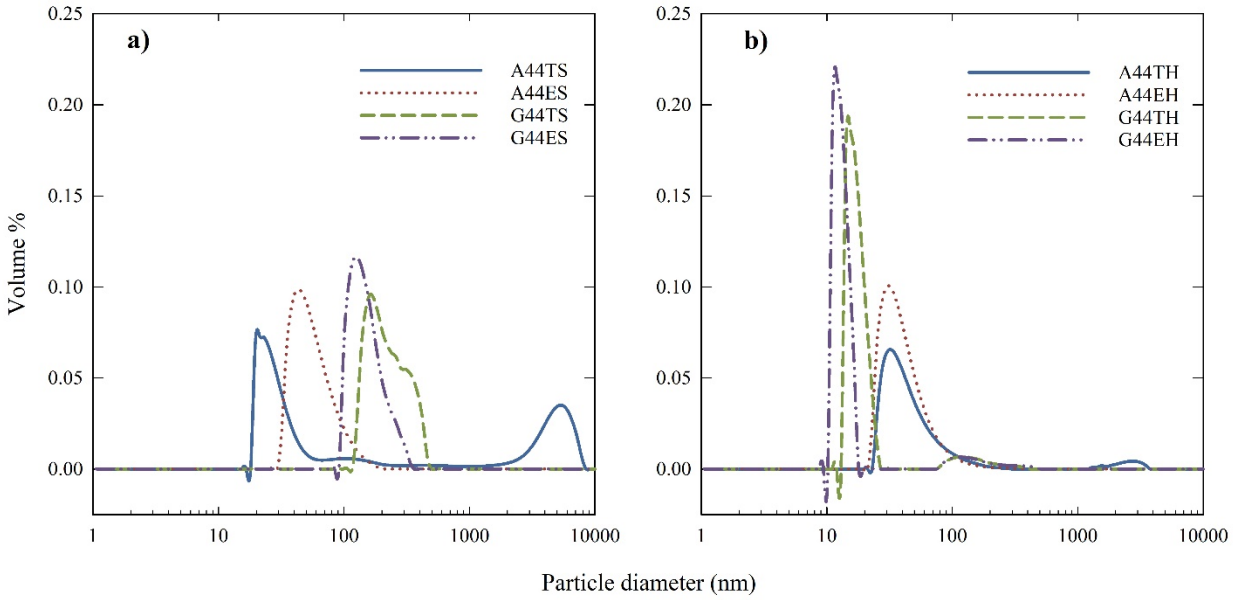

Figure 5.2 Effect of a) primary (S) and b) secondary (H) homogenization, on the particle size distribution of egg yolk/fish oil triglycerides (T) or ethyl esters (E) emulsions containing gum arabic (A) or gum guar (G). Each curve is the average of three replications. All treatments showed a monomodal particle size distribution in the nano-size region after high-pressure homogenization, with exception of A44TH, which showed some residual in the micro-size region.

5.4.3.2 Average particle size

The factor that caused the highest variability on the average particle size was the esterification type. For instance, the mean particle size of emulsions containing triglycerides was significantly larger than with ethyl esters at all levels evaluated. The highest effect of time on the average particle size was observed at day 10, whereas the treatments most affected by time where those containing gum arabic.

The effects of processing and time on the particle size of emulsions are shown in Figures 5.3a and 5.3b, when gum arabic was used as secondary layer, and Figure 5.3c and 5.3d, when gum guar was used. At day 0, the smallest average particle size corresponded to the Control-AS (72.4 nm); however, this value was not significantly different from the other controls (-AH, -GS, and -GH). On the other hand, a significantly larger particle size was obtained in emulsions

carrying triglycerides. Samples subjected to secondary homogenization (high-pressure homogenization at 200 MPa) showed significantly lower particle size ($p < 0.01$) than their counterparts processed only through primary homogenization. High-pressure homogenization process has shown to form emulsion with smaller particle size, usually in the nano-size range, regardless of the biopolymer used as coating material. For instance, using high-pressure homogenization of sodium caseinate-coated fish oil emulsions at 12 kpsi showed an average particle diameter of 168 nm (Chang et al., 2016). The strongest effect of high-pressure homogenization was observed in samples containing gum arabic and 22 or 44% triglycerides after secondary homogenization (A22TH and A44TH), for which about 5 and 12-fold reduction in size, respectively, was obtained compared to those processed by primary homogenization (A22TS and A44TS). The significantly larger particle size obtained for samples A22TS and A44TS could be the result of bridging flocculation (Xu et al, 2017). This effect is observed when the concentration of molecules of the polysaccharide is not enough to cover the entire droplet, causing one molecule to adsorb to the surface of several droplets at the same time. Xu et al (2017) observed the same effect in emulsions using rice glutelin as primary coating and 0.01% of anionic polysaccharides as the secondary layer. Therefore, our results suggest that in order to have emulsions with better stability, higher concentrations of gum arabic are needed. Compared to other surface-active biopolymers, gum arabic displays a lower affinity for oil-in-water interfaces, a ratio as high as of 1:1 gum arabic: oil phase ratio to form emulsions more resistant to flocculation (McClements, 2016).

Nevertheless, this was not the case for emulsions where gum guar was used, since the particle size and distribution did not show this bridging effect. In chapter 3 we discussed the significant effect of storage time on the particle size of emulsions using raw and phospholipase-1 treated egg yolk under accelerated shelf-life conditions. However, in this study, treatments using gum guar did not seem to be significantly affected by time at the same conditions (45 °C, 10 days of storage). It has been shown that polysaccharides can form thick interfacial layers, which could prevent droplet flocculation and coalescence while globular proteins alone can prevent coalescence but not flocculation (Chanamai et al., 2002, Charoen et al., 2011). This effect was shown by the stability of emulsions containing gum guar. The effectiveness of gum guar, a nonionic polysaccharide, to prevent aggregation is the result of the hydration of its polar head groups. The relatively high temperature used in this study for long term, caused dehydration of

these head groups eventually resulting in aggregation (McClements, 2016), as observed by the increase in average particle size. Therefore, a significant effect of storage on the particle size of emulsions stabilized by gum guar was observed after 10 days.

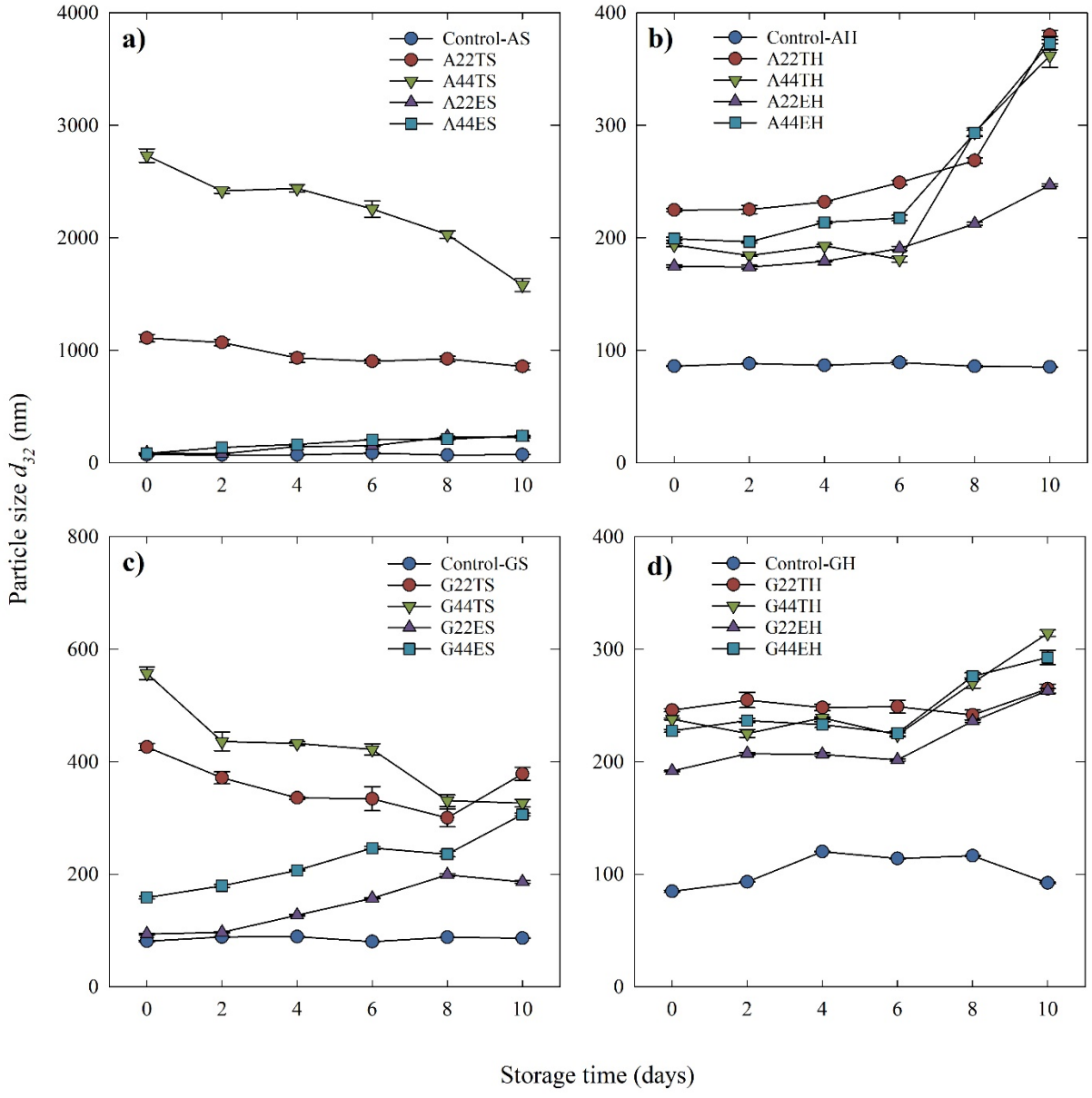

Figure 5.3 Average particle size of egg yolk/fish oil triglycerides (T) or ethyl esters (E) emulsions containing gum arabic (A) after a) primary and b) secondary homogenization; or gum guar (G) after c) primary and d) secondary homogenization. Changes in the particle size are shown over 10 days of storage at 45 °C. The graphs are organized to display differences between main plots (polysaccharide type, rows) and the influence of the split-plot (processing type, columns). Each data point corresponds to the mean ± standard error of three replications.

A significant effect ($p < 0.05$) of the EPA+DHA concentration was also observed. At lower concentrations of the lipid phase, a smaller average particle size was recorded. Lower concentrations of EPA+DHA added, means higher wall material: oil phase ratio. The resulting smaller particle size could be explained by a higher availability and faster adsorption of the biopolymer molecules, used as emulsifiers, to the oil phase surface (Bai et al, 2016), resulting in more efficiency to coat the lipid droplets and form smaller particles.

5.4.4 Influence of treatment on the EPA+DHA encapsulation efficiency of egg yolk

Polysaccharides have shown potential to enhance the stability of oil-in-water emulsions (Ercelebi and Ibanoglu, 2009; Chivero et al, 2015; Xu et al, 2017). In addition to particle size changes, the stability of the emulsions can also be evaluated by the release of the core material over time. Therefore, the influence of adding anionic (gum arabic) and neutral (gum guar) polysaccharides on the encapsulation efficiency of egg yolk/fish oil emulsions was evaluated under accelerated shelf-life conditions (up to 10 days at 45 °C). Table 5.1 shows the initial encapsulation efficiency and the total EPA+DHA that remained encapsulated after 10 days of storage at 45 °C, for emulsions with the highest concentrations of EPA+DHA. At day 0, the encapsulation efficiency of emulsions containing gum guar that were produced by secondary homogenization (G44TH and G44EH) was significantly higher than those using a combination of gum arabic and primary homogenization (A44ES and A44TS).

The lack of differences between the two main plots in our design (gum arabic versus gum guar as secondary layer) indicate that despite the bridging effect observed in treatments with gum arabic, the core material remained encapsulated within the continuous phase of the emulsion. Nevertheless, differences on the efficiency between the two polysaccharides were clearly observed after 10 days of storage, when the analysis of variance showed a highly significant influence ($p < 0.001$) of treatment, time and their interaction on the percentage of encapsulated oil, displaying a clear separation of the main plots. For instance, the four samples prepared with gum guar showed significantly higher ($p < 0.05$) encapsulation efficiency than that of gum arabic.

Table 5.1 Encapsulation efficiency of emulsions containing 44% EPA+DHA (w/w egg yolk dry matter basis) and gum arabic (A) or gum guar (G), after primary (S) and secondary (H) homogenization and remaining encapsulated oil after 10 days of storage at 45 °C. The percentages are given based on total EPA+DHA quantification. Every value corresponds to the mean ± standard error of three replications. Different letters within columns mean significant differences among treatments. The encapsulated oil was significantly lower for all treatments at day 10. Significance was considered at $p < 0.05$.

Treatment	Encapsulation Efficiency (%) at Day 0	Encapsulated oil (%) at Day 10
A44TS	97.3 ± 0.4^{b}	80.7 ± 0.7^{d}
A44TH	98.4 ± 0.2^{ab}	89.7 ± 0.3^{c}
A44ES	97.0 ± 0.2^{b}	91.0 ± 0.7^{c}
A44EH	98.4 ± 0.2^{ab}	90.6 ± 0.5^{c}
G44TS	98.0 ± 0.2^{ab}	94.7 ± 0.2^{a}
G44TH	$99.5 \pm .1^{a}$	94.6 ± 0.3^{a}
G44ES	97.4 ± 0.1^{b}	94.6 ± 0.4^{a}
G44EH	$99.4 \pm .1^{a}$	92.8 ± 0.3^{b}

These results could be partially attributed to the behavior of gum arabic under the pH conditions used during the processing of the emulsions. The initial egg yolk solutions were adjusted to pH 6.0 before proceeding with primary homogenization. Under these conditions, the zeta potential of egg yolk is slightly negative (Navidghasemizad et al., 2015), which could have caused repulsion between the gum arabic molecules and the proteins adsorbed at the surface of the lipid droplets, resulting in poor efficiency to form a secondary layer (Chang et al., 2016). Also, as discussed in section 5.3.2, gum arabic requires a ratio of 1:1 (w/w) with the oil phase in order to reach its stable effect. Results from the encapsulation efficiency over time from this study seemed to be related with the overall particle stability found for the same treatments.

Furthermore, the effect of processing time was significant only in emulsions using gum arabic as secondary layer after 10 days of storage, being those emulsions formed by secondary homogenization the ones displaying the lowest release of the core material. The lack of significant differences between processing type on emulsions containing gum guar suggests that stability of the emulsions at this stage was given mostly by the ability of the polysaccharide to

act as an extra barrier to entrap the lipids in the core material and not due to the particle size of the emulsion.

5.4.5 Oxidative stability of emulsions under accelerated shelf-life conditions

In order to assess the oxidative stability of samples under accelerated shelf-life conditions, the propanal content and peroxide value were measured. These analyses were conducted in emulsions with the highest EPA+DHA content at 0, 2, 4, 6, 8, and 10 days of storage at 45 °C.

The propanal and peroxide formation in the emulsions during storage is shown in Figure 5.4. The effect of the fixed factors on the oxidative stability of emulsions showed the same significance as their effects on encapsulated oil. These results were expected, as it is known that the oil released from the interior of the particles will be more prone to oxidation in the presence of oxygen and high temperatures (Duan et al., 2011; Ramakrishnan et al., 2013; Alvarez et al., 2014).

Emulsions using gum guar (Fig. 5d) had higher stability as shown by the lack of significant peroxide formation from day 0 to day 4 of storage whereas those emulsions using gum arabic (Fig. 5b) showed significantly higher values on day 2 of storage. Overall, a clear difference was observed between main plots, with lower peroxide values for treatments using gum guar throughout the storage time evaluated. At day 10, emulsions containing triglycerides, regardless the processing type, showed significantly lower peroxide values than those containing ethyl esters.

A similar pattern was observed for propanal formation. No propanal formation was detected on emulsions freshly prepared (day 0); whereas at the end of the storage time (day 10), the lowest (α= 0.05) propanal contents, with values of 4.85 and 4.92 μg/g emulsion, were obtained from emulsions containing gum guar and triglycerides, G44TH and G44TS, respectively. The processing used for emulsion formation did not show a significant effect on the oxidative stability of the emulsions over the evaluated storage period. This result follows the same pattern as the one observed on the encapsulation efficiency. The lack of significant effects of processing type on the oxidative stability emulsions containing gum guar, suggests that the stability of the emulsions at this stage was given mostly by the ability of the polysaccharide to act as a barrier between the environment and the core material.

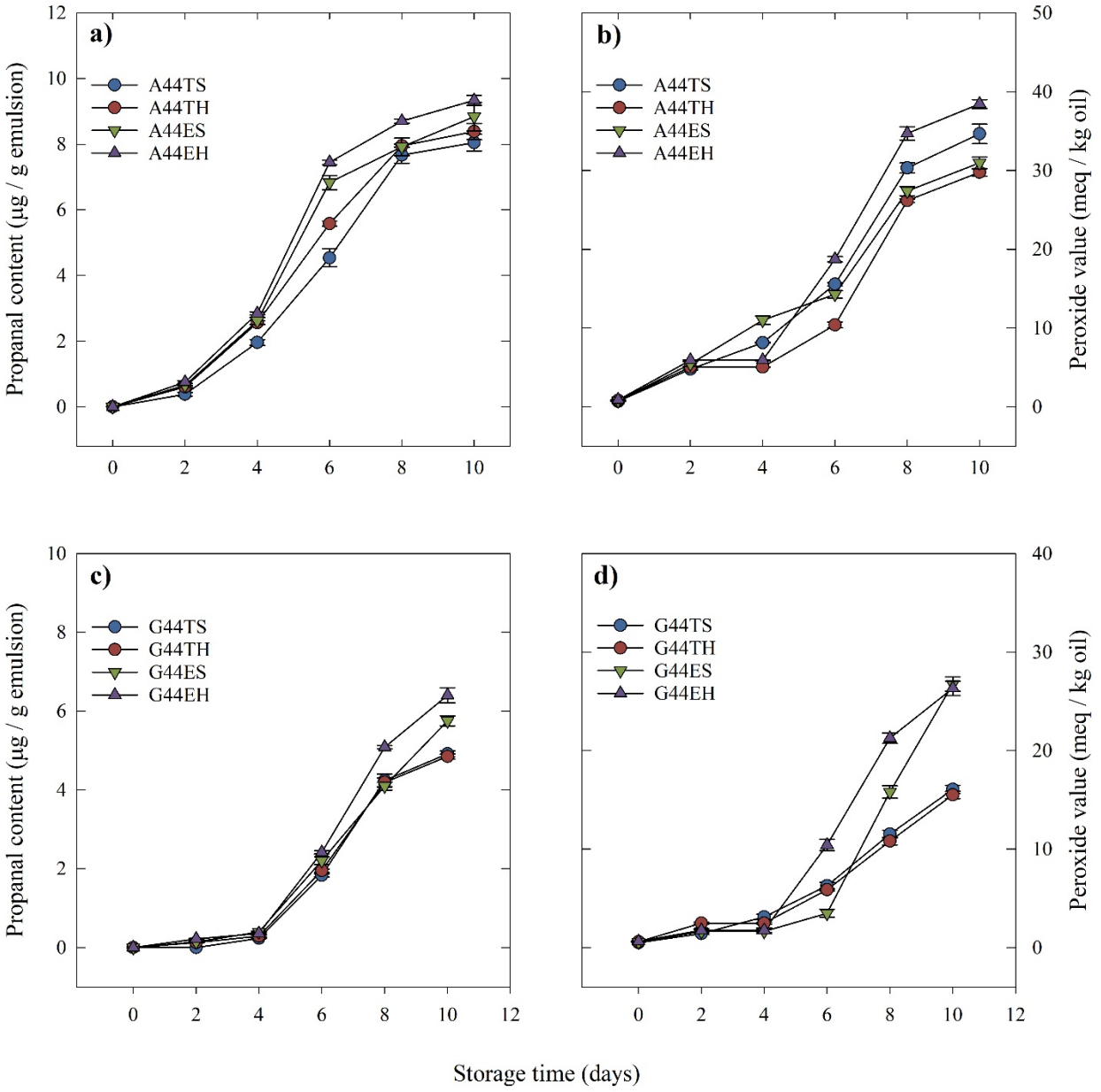

Figure 5.4 Oxidative stability of egg yolk/fish oil triglycerides (T) or ethyl esters (E) emulsions containing gum arabic (A) after a) primary and b) secondary homogenization; or gum guar (G) after c) primary and d) secondary homogenization. Propanal and peroxide contents are shown over 10 days of storage at 45 °C. The graphs are organized to display differences between main plots (polysaccharide type, rows) and the influence of the split-plot (processing type, columns). Each data point corresponds to the mean ± standard error of three replications.

5.4.6 Effect of treatment on the toxicity of the emulsions under accelerated shelf-life conditions

It has been shown that the oxidation of long chain polyunsaturated fatty acids produces toxic compounds related to aging, mutagenesis and carcinogenesis (Nair et al., 1986, Nicolson and Ash, 2014).

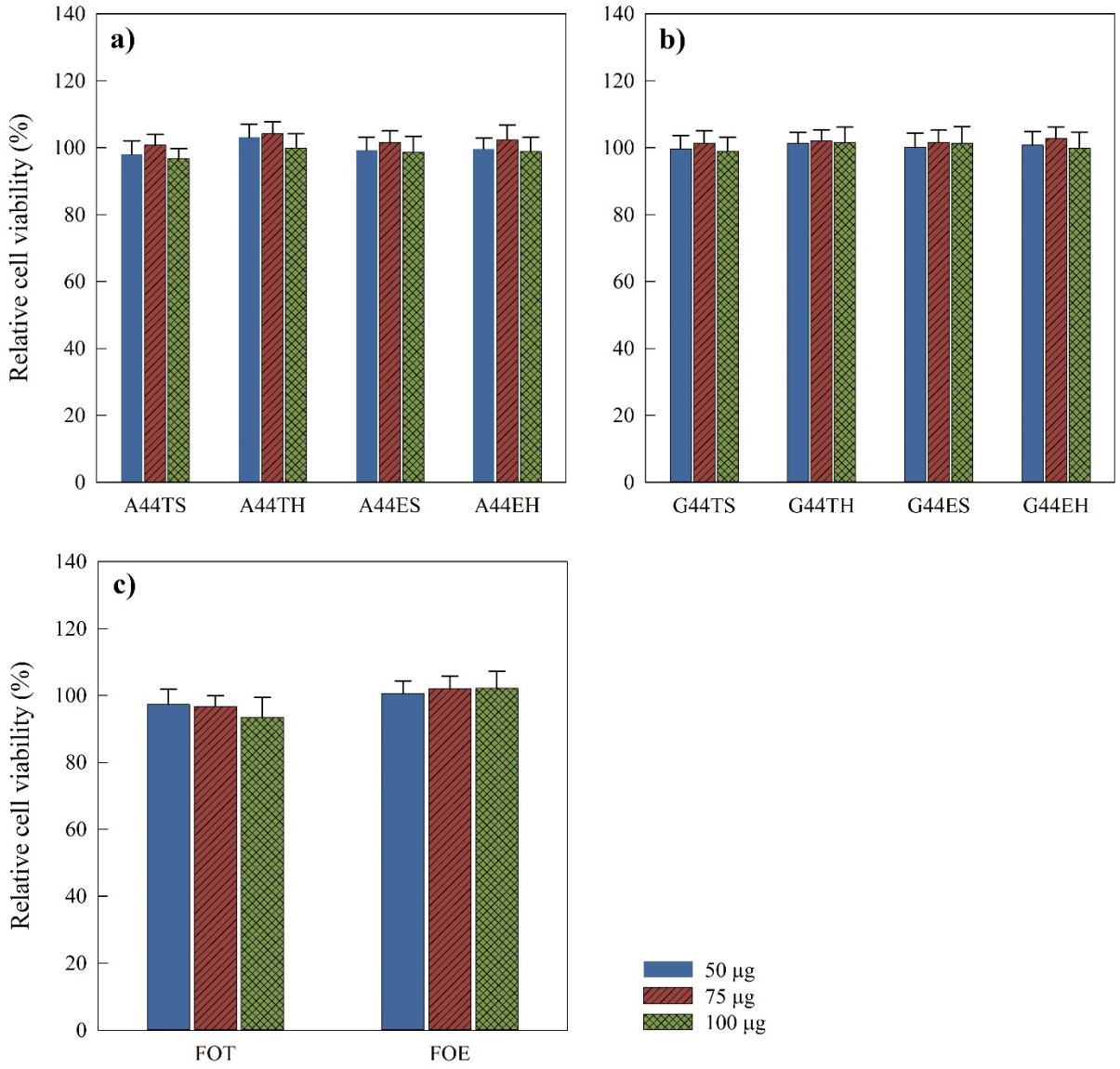

Figure 5.5 Toxicity of egg yolk/fish oil triglycerides (T) or ethyl esters (E) emulsions containing gum arabic (A) or gum guar (G) formed by primary (S) and secondary (H) homogenization, and the non-encapsulated fish oil triglycerides or ethyl esters controls (FOT and FOE, respectively) at 3 different concentrations (µg fish oil / mL medium) at day 0. Each bar represents the mean ± standard error of four to six replications. No significant differences with the blank (medium without emulsions) were found at $\alpha = 0.05$.

In order to complete the study on the effectiveness of polysaccharides used as secondary layers in egg yolk/fish oil emulsions, the viability of Caco2 cells incubated with the emulsions at different stages of storage was evaluated. To assess the toxicity of the emulsions, the mitochondrial enzymatic activity was measured using an MTT-based assay.

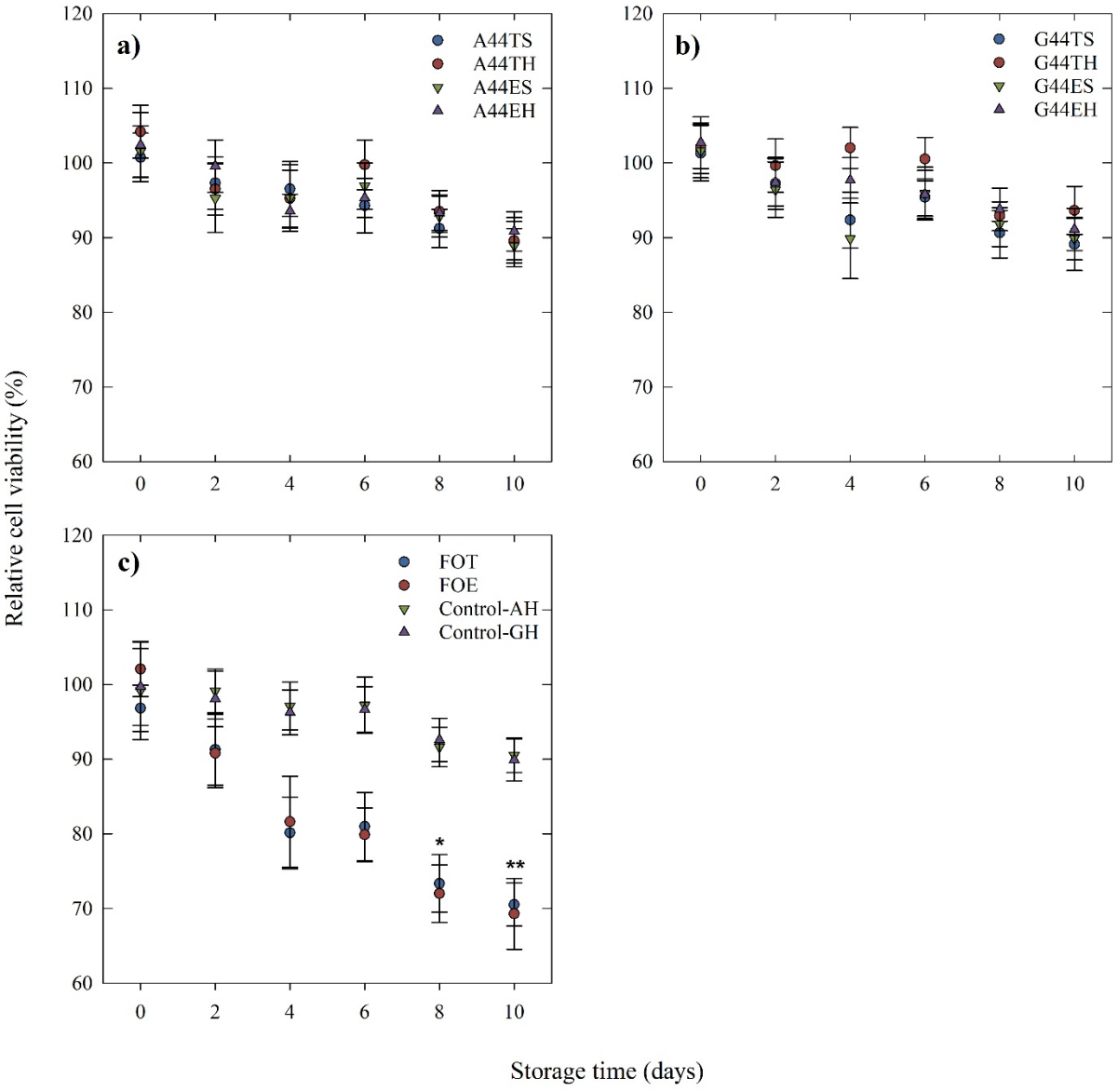

Figure 5.6 Toxicity of egg yolk/fish oil triglycerides (T) or ethyl esters (E) emulsions containing gum arabic (A) or gum guar (G) formed by primary (S) and secondary (H) homogenization, and the non-encapsulated fish oil triglycerides or ethyl esters controls (FOT and FOE, respectively), over 10 days of storage at 45 °C. All samples were tested at a concentration of 75 μg fish oil/mL medium. Each point represents the mean of four to six replications ± standard error.

The toxicity of fresh emulsions (day 0) and the negative controls was evaluated at different concentrations (Figure 5.5). This analysis was conducted in order to screen for potential dose-dependent toxicity. No significant differences in cell viability were found within treatments at all levels evaluated. However, lower variation was overall observed at a dose of 75 μg fish oil/mL medium; therefore, this concentration was used for analyses over time.

The toxicity of the emulsions, the controls without fish oil, and fish oil without encapsulation over 10 days of storage at 45 °C is shown in Figure 5.6. Significant differences in cell viability were observed only for the negative controls, resulting in an average viability of 70.47% (previously reported in chapter 2) for fish oil triglycerides (FOT) and 69.52% for ethyl esters (FOE) (Fig. 5.6c). These results show the positive effect of adding polysaccharides to egg yolk/fish oil emulsions on the development of cytotoxic components. Further studies are needed to evaluate the mechanisms with which these polysaccharides exert these beneficial properties.

5.5 Conclusions

In this study, egg yolk was used as the primary emulsifier while gum arabic or guar were used to form a secondary layer by primary or secondary homogenization. Secondary homogenization, fish oil triglycerides, and gum guar significantly increased the apparent viscosity of the emulsions. The distribution curves of samples using gum guar as the secondary layer showed one main peak in the nano-size region, whereas gum arabic showed an important second peak in the micro-size region. The particle size of emulsions using gum arabic was most affected by time. At the polysaccharide concentrations tested, gum guar was more resistant to flocculation than gum arabic. Emulsions formed with gum guar and secondary homogenization had higher encapsulation efficiency than the other treatments. Moreover, after 10 days of storage, treatments using gum guar contained less surface oil than their counterparts using gum arabic. Emulsions using gum guar had higher oxidative stability as shown by the lack of significant peroxide and propanal formation from day 0 to day 4 of storage whereas those emulsions using gum arabic showed significantly higher values on day 2 of storage. Additionally, significant differences in cell viability were observed only for the negative controls, while emulsions containing the same concentrations of EPA+DHA did not show toxicity at the conditions evaluated. Overall, egg yolk/gum guar emulsions were more stable and more efficient to protect EPA+DHA against oxidation than those using gum arabic.

CHAPTER 6: Consumer acceptance of fortified food products using egg yolk-based emulsions as delivery systems of EPA+DHA

6.1 Abstract

For any newly developed food product, consumer acceptance is the most important factor for its success. The main objective of this study was to evaluate the stability and consumer acceptance of two food products that have been fortified with 250 mg eicosapentaenoic and docosahexaenoic acids (EPA+DHA) per 90 g from three different egg yolk-based emulsions and a non-encapsulated control. The stability of the emulsions was evaluated at 60 °C and after freeze-thawing. Moreover, a 9-point hedonic scale was used to assess consumer acceptance. PLA_1-treated egg yolk and egg yolk/gum guar emulsions had the lowest surface oil content and lowest degradation (1.3 and 1.5%, respectively) of EPA+DHA at 60 °C. In addition, egg yolk/gum guar emulsion was the most resistant against freeze-thawing. EPA+DHA degradation at processing stage was significant only for ice cream samples using non-encapsulated fish oil. However, the EPA+DHA content reduced significantly in all cake samples after baking but samples containing emulsions were not significantly different from those using non-encapsulated fish oil. Scores for flavor, aroma, and overall liking of ice cream showed that non-encapsulated controls were the least liked, scoring the lowest for flavor liking at only 5.0, with consumers noting “bad flavor”, “fishy taste”, “unpleasant aftertaste”, and “rancid notes”. Furthermore, consumer acceptance of cake did not show significant differences among treatments, with average scores for flavor and aroma liking ranging from 6.2-6.4 and 6.3-6.8, respectively. These results showed that egg yolk can be used as a whole food matrix, after pre-treated with phospholipase A_1, or along with polysaccharides, to prevent the development of off flavors and unpleasant aromas caused by EPA+DHA oxidation.

6.2 Introduction

Our previous works have assessed the efficiency of several egg yolk-based emulsion formulations to encapsulate and prevent oxidation of long chain *n*-3 polyunsaturated fatty acids, focusing on eicosapentaenoic and docosahexaenoic acids. Emulsion-based delivery systems should have excellent stability during the emulsion formation and throughout processing and storage of the final food product. Nevertheless, when such emulsions are being developed for further use as delivery systems for food applications, the consumer acceptance of the final food product should be assessed. The physicochemical characteristics of the original emulsion should be taken into account since they could impact the overall quality of the final product.

The success of a newly developed food product will greatly depend whether the final consumer likes it or not (Le and Worch, 2015). Consequently, consumer acceptability tests are performed to evaluate the potential success of a product. Consumer acceptance and preference can be assessed using untrained panelists, where the panelist is a regular consumer of the product being evaluated. An affective test is used for this purpose to describe how well a product is liked or which product is preferred between two or more choices. The 9-point hedonic scale (Sjostrom, 1954) is the most widely used scale to assess the degree of liking or disliking of a product.

A typical food system is composed of different surface-active components interacting with each other, such as proteins, polysaccharides, and phospholipids. These interactions can eventually lead to destabilization of the whole system. Moreover, environmental stresses, such as variations in temperature, pressure, and mechanical agitation, encountered during food processing and storage can contribute to modifications in the original structure of the product (McClements, 2016). These modifications are mostly desired; however, they could bring issues such as oxidation of lipids and proteins, hydrolysis of proteins or polysaccharides, and cross-linking of proteins (Belitz et al., 2009). Therefore, fortifying food products with *n*-3 fatty acids is usually a challenging task, due to the high susceptibility of these compounds to such environmental stresses that result in a first stage in the formation of undesirable flavor molecules and later on the formation of toxic compounds.

The sensory attributes of *n*-3 fortified food products have been extensively tested. For instance, in a study conducted by Hayat et al. (2014) it was found that nutrient-enriched eggs, produced by feed manipulation, were accepted by the consumers showing similar overall quality with regular eggs; significant differences were obtained only for the egg yolk color. However,

the number of panelists (n= 10) was low. In another study, Coorey et al. (2015) reported no significant changes in consumer preference of table eggs from hens with supplemented diets (30% chia flour) in comparison with the control eggs. Nevertheless, they observed a decrease in the overall consumer acceptability when supplementing the diets with 30% flaxseed flour and 40% chia seed flour. Furthermore, Chee et al (2005) evaluated the effect of the addition of 500 mg *n*-3 fatty acids from an algae oil emulsion (before and after homogenization) on the sensory quality of strawberry flavored yogurt. Trained panelists detected a strong fishy flavor in both treatments; whereas untrained consumer panelists rated the treatments as they did the control (without added *n*-3).

Considering the information presented above and the stability results of the emulsions reported in the previous chapters of this thesis, we hypothesize that the consumer acceptability of food products fortified with *n*-3 fatty acids will be improved using egg yolk-based emulsions as delivery systems, compared to non-encapsulated fish oil. Thus, the main objective of this study was to evaluate the stability as well as the sensory characteristics of two different food products that have been fortified with EPA+DHA using three different egg yolk-based emulsions as delivery systems.

6.3 Materials and methods

6.3.1 Materials

Eggs used for emulsion and food preparation were purchased from a local grocery store (Superstore, Edmonton, CA) and used within 3 days of storage at 4–6 °C. Egg yolks were separated manually from the whites and carefully placed onto paper towels to absorb excess albumen. The vitelline membrane was then punctured with a spatula and the egg yolk content was collected in a beaker placed in an ice bath. The collected egg yolk was then pasteurized at 61.1 °C for 3.5 min according to the Canada Processed Egg Regulations (Agriculture and Agri-Food Canada, 2018) for eggs of human consumption.

Food grade fish oil from wild Alaska Pollock (*Gadus chalcogrammus*) was purchased from a commercial brand and used as source of EPA+DHA. As stated in the product label, the fish oil contained 55% EPA and 20% DHA as triglycerides. Food-grade Lecitase® Ultra (phospholipase-A1, activity= 10 KLU/g) was obtained from Novozymes (Bagsvaerd, Denmark). Food-grade gum guar was purchased from a local grocery store (Planet Organic, Edmonton,

Canada). All the ingredients and consumables for the sensory study were purchased from a local grocery store (Superstore, Edmonton, CA). All reagents used for chemical analyses were obtained from Sigma-Aldrich (Oakville, ON, Canada).

6.3.2 Egg yolk pre-treatment

The processes described below, with the exception of total and surface oil and EPA/DHA quantification, where conducted in a food-grade laboratory at the Agriculture and Forestry building of the University of Alberta (Edmonton, Alberta, CA).

6.3.2.1 Egg yolk solutions preparation

Three aliquots of freshly collected egg yolk were taken to determine the moisture content in a convection oven set a 105–110 °C for 5 h. The egg yolk was then adjusted to 30% solids with potable water, for egg yolk (EY) and PLA_1-modified egg yolk (PLA_1-EY) emulsions. For the polysaccharide/egg yolk emulsions (G-EY), the collected egg yolk was used directly to form a pre-emulsion then mixed with a gum guar solution as described in section 5.2.3. Dilution of the egg yolk was conducted to facilitate the enzymatic reaction with Lecitase® Ultra and was applied to the egg yolk and egg yolk/polysaccharide samples to make comparison of the chemical analyses.

6.3.2.2 Enzymatic treatment using phospholipase A_1

The egg yolk solution was placed in a jacketed beaker with gently mixing and let settle to a constant temperature of 50 °C. The initial pH of the solution was adjusted to 6±0.01, followed by addition of 0.05% (w/w on dry matter basis) Lecitase® Ultra. The reaction was allowed for 5 hours keeping the pH constant at 6±0.1. The enzymatic reaction was stopped by heating the samples at 72 °C for 5 min. PLA_1-treated egg yolk samples were kept at 4–6 °C and used to prepare the emulsions within 24 hours.

6.3.3 Emulsion formation by secondary homogenization

Fish oil was added to EY and PLA_1-EY solutions, or whole egg yolk (egg yolk without solids adjustment) to a final ratio of 1:1 fish oil: egg yolk solids, then pre-homogenized at 14,000 rpm for 1 min. The whole egg yolk emulsion was further mixed with a gum guar solution to a final

concentration of 30% (w/w) egg yolk solids and 0.25% (w/w) gum guar at 400 rpm for 4 min. The resulting primary emulsions were then homogenized at 200 MPa in a Pressure Cell Homogenizer FPG 12800 (Stansted Fluid Power LTD, London, UK) with a hydraulic relief pressure of 240 MPa. Each treatment was divided into individual food-grade airtight plastic containers and kept at 4–6 °C for no more than 48 h for further use.

6.3.4 Stability of emulsions under harsh temperature conditions

The surface oil and total EPA+DHA were quantified in samples after incubation at 60 °C for 2 hours, and in thawed samples after freezing at –20 °C for 12 h. Non-encapsulated fish oil was subjected to the same conditions and used as control.

6.3.4.1 Total and surface oil

EPA+DHA in total oil was extracted using the method proposed by Bligh and Dyer (1959). A full description of the extraction process and quantification is given in Chapter 2, section 2.3.6.1. The extraction and quantification of surface oil was performed according to Chapter 2 section 2.3.6.2.

6.3.4.2 Methylation and EPA+DHA quantification

The methylation and quantification of EPA+DHA was conducted following the method developed by Joseph and Ackman (1992), using tricosanoic acid (23:0) methyl esters as internal standard. EPA+DHA triglycerides were quantified as described in Chapter 2, section 2.3.6.3.

6.3.5 Food products preparation

Two different food products, ice cream and cake, were prepared to assess the stability of the emulsions under different processing and storage conditions. A proportion of the egg yolk originally included as part of their formulation was substituted with the emulsions to achieve a theoretical concentration in the final food product of 250 mg EPA+DHA per portion (90g). In the case of the control treatment (FO), a proportion of the butter was substituted with the direct addition of fish oil to keep the fat proportions equal. Standard recipes for each food product were elaborated by comparing recipes from online resources and then scaled to laboratory conditions.

6.3.5.1 Vanilla ice cream

372 g whole milk (3.25% w/w fat), 405 g fresh cream (40% w/w fat), 1g salt and 60 g sugar, were whisked in an electric mixer until homogeneous (Mixture 1). Pasteurized egg yolk plus the corresponding proportion of EY, PLA_1-EY, or G-EY emulsions were mixed. 92 g of the egg yolk mixture, 10 g natural vanilla extract, and 60 g sugar were mechanically mixed in a medium bowl until the mixture was pale and thick (Mixture 2). Mixture 1 was heated to about 80 °C in a medium saucepan set over medium-low heat then Mixture 2 was added slowly under constant stirring and heated to 86 °C for 3 – 4 min to allow the final mixture to thicken enough to cover the back of a wooden spoon. This final mixture, the ice cream base, was cooled down to room temperature then poured through a fine mesh onto an airtight container, covered, and refrigerated for 12 hours. After this period, the ice cream base was churned for 25 min in a Cuisinart ICE-70C ice cream maker. The ice cream was stored in an airtight container at -20 °C for 12–24 h, until served.

6.3.5.2 Vanilla cake

336 g white all-purpose wheat flour, 4.6 g baking powder, and 1.4 g salt were mixed in a medium bowl (Mixture 1). 154 g butter and 118 g sugar were whisked in an electric mixer at medium speed until homogeneous (Mixture 2). Pasteurized egg yolk and a proportion of EY, PLA_1-EY, or G-EY emulsions were mixed then added with 90 g egg white and 16 g vanilla extract (Mixture 3). Mixture 3 was then added to Mixture 2 and continued whisking until homogeneous. One third of Mixture 1 was then added followed by half of the milk (112 g). This procedure was repeated, ending with the last third of Mixture 1. The batter was placed in round cake pans previously coated with butter and baked in an electric oven at 177 °C for 32 minutes, then cooled down to room temperature and stored in zipper-sealed plastic bags at 4–6 °C for up to 12 h until served.

6.3.6 Sensory analysis

Sensory evaluation of the designated food product with its three formulations was conducted over two different sessions. In order to assess the effectiveness of the carriers, non-encapsulated fish oil was added to the food product formulation under the same processing conditions to serve as a negative control.

Each product tasting was conducted from 10 am to 2:30 pm at the sensory evaluation room located in the Agriculture/Forestry Centre at the University of Alberta. A total of 108 panelists were recruited from staff, students, and visitors of the University of Alberta through e-mail (using departmental listservs) and flyers. In order to participate, subjects were required to be +18 years old, not present any allergies to the ingredients, and consume the designated food product at least once a month. All panelists were asked to sign a consent form prior to the test confirming they were aware of the benefits and risks of participating in the study. The sensory test was reviewed and approved by the University of Alberta Research Ethics Board 2, under the project name "Consumer preference of omega-3 fortified food products", ID. Pro00065902_AME1, on January 8th, 2019.

To begin the test, participants were asked to sit in individual sensory booths with white light and were then presented with a tray consisting of four samples of the same product (3 treatments and 1 negative control) labeled with random three-digit codes. 10–12 g ice cream was served in 30 mL clear plastic containers with clear plastic lid and a spoon. 10–12 g cake slices were served at room temperature in 60 mL clear plastic containers with clear plastic lid and a spoon. Each panelist evaluated all four randomly ordered samples. Trays also contained a cracker and potable water to clean their palates, a napkin, a pencil, and a questionnaire. The questionnaire required demographic information such as gender and age and included detailed instructions to assess each sample.

Participants assessed their degree of liking or disliking of each sample in terms of appearance, flavor, aroma, texture, and overall opinion using a 9-point hedonic scale with a centered neutral category as follows: 1= dislike extremely, 2= dislike very much, 3= dislike moderately, 4= dislike slightly, 5= neither like nor dislike, 6= like slightly, 7= like moderately, 8= like very much, and 9= like extremely (Lawless and Heymann, 2010). Participants were also provided with open space for comments about the least liked sample.

6.3.7 EPA+DHA content in food products

Total EPA+DHA was quantified at different stages of the food processing to assess the efficiency of the emulsions. EPA+DHA was quantified from the total oil extracted from the ice cream base, cake batter, freshly cooked ice cream and cake, and after 10 days of storage at -20 and 4–6 °C for ice cream and cake, respectively.

6.3.8 Statistical analysis

The chemical analyses of emulsions followed a 4x2x2 Complete Block Design with three replications, where the independent factors were the carrier at four levels, the storage temperature at 2 levels, and finally, the sampling time (as a fixed factor) was evaluated at 2 levels. Moreover, a 2x4x3 Complete Block Design was used for the chemical analyses of food products during processing and storage, with two levels of food products, 4 levels of treatment, and 3 levels of sampling stage. These results were expressed as the mean and the standard error. A multiple analysis of variance (MANOVA) was used to find significant factors over time and a one-way analysis of variance (ANOVA) was used to analyze differences at one given time. Significant differences of the mean values were considered at $p < 0.05$ using Tukey test.

The consumer and product effect on the food products' acceptance was analyzed by one-way ANOVA using the AovSum and the plot.PCA functions from the SensoMineR package. The one-way ANOVA was performed across treatments for each sensory attribute of each product. The consumer acceptance of vanilla ice cream was assessed from a product perspective, whereas the vanilla cake was analyzed from a consumer perspective as no significant differences were observed from the product analysis. Additionally, a univariate approach using the Fischer's LSD test with Bonferroni p-value adjustment was used to find the best-liked products according to their flavor, aroma, and overall liking scores. All statistical analyses were conducted using the statistical package R version 3.3.2 in R studio version 1.0.136 (R Core Team, 2016).

6.4 Results and Discussion

In the present work, three egg yolk-based emulsions, raw egg yolk (R), PLA_1-treated egg yolk (PLA_1-EY), and gum guar/raw egg yolk (G-EY), were used as delivery systems of fish oil, containing 55% EPA and 25% DHA (w/w), in food products. The efficiency of the treatments was assessed by their ability to prevent degradation of EPA+DHA at different stages of food processing and storage. The emulsion treatments were selected due to the potential to protect EPA+DHA from oxidation, as shown in Chapters 3 to 5 of this thesis. Egg yolk without further treatment had proven to be an efficient carrier of EPA+DHA; however, PLA_1-treated egg yolk showed improved stability at high temperature conditions. Moreover, from Chapter 5, egg yolk/gum guar emulsions showed to enhanced encapsulation efficiency and oxidative stability under accelerated shelf-life conditions.

6.4.1 Surface oil and total EPA+DHA in emulsions under different storage conditions

The EPA+DHA content in the fish oil used in this work was re-tested in our facilities to adjust for any handling variations. The theoretical initial content was 17.13±0.11 g EPA+DHA/100 g of emulsions. This data point was used as 100% reference for the degradation analysis.

6.4.1.1 Surface oil

The conditions 60 °C for 2 hours was selected to simulate harsh temperatures used during food processing. Commercial freezing conditions (-20 °C) were used to evaluate the behaviour of emulsions during a freeze-thaw cycle. The treatments and their interaction with temperature had a highly significant influence ($p < 0.001$) on the surface oil of the emulsions.

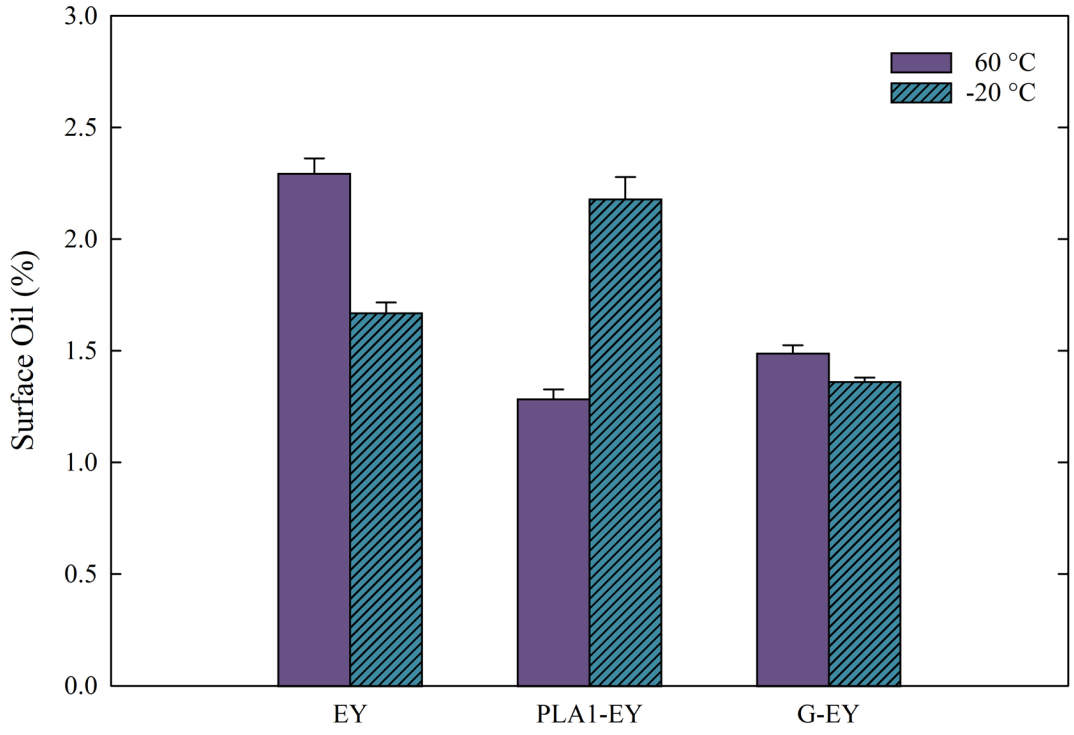

Figure 6.1 Percentage of surface oil in emulsions after 2 hours of incubation at 60 °C and after freeze-thawing. The x axis refers to the treatments egg yolk (EY), phospholipase A_1-treated egg yolk (PLA_1-EY), and egg yolk/gum guar emulsions (G-EY). All treatments were significantly higher than 0. Different letters mean significant differences among treatments under the same conditions. An $\alpha = 0.05$ was set to assess significant differences.

As seen in Figure 6.1, PLA_1-EY and G-EY had the lowest content of surface oil at 60 °C, with averages of 1.3 and 1.5%, respectively; whereas EY samples were significantly lower with an average of 2.3%. Moreover, after freeze-thaw cycling, all samples were significantly different among each other, being G-EY the most resistant (1.4%) and PLA_1-EY the less resistant (2.2%). Protein/polysaccharide emulsions have shown to endure a wider range of environmental stresses than proteins alone, such as high temperatures and freeze-thaw cycling (McClements, 2016). In this study, egg yolk/gum guar emulsions (G-EY), were found the most stable under high temperatures and freeze-thaw cycling as shown by the lack of significant differences in surface oil at both conditions.

6.4.1.2 EPA+DHA degradation

The degradation of the original EPA+DHA content in non-encapsulated fish oil and emulsions after harsh temperature conditions is shown in Figure 6.2. Treatment, temperature, and their interaction had a significant influence on the EPA+DHA content of the emulsions and the fish oil control.

The EPA+DHA content in all samples changed significantly depending on the temperature conditions. At 60 °C, PLA_1-EY and G-EY emulsions had the lowest degradation of EPA+DHA, whereas FO had the highest with 2.166% degradation. These results followed the same trend found for surface oil, which is in agreement with previous findings that the oil released from the core of the particles to its surface is more prone to oxidation (Duan et al., 2011; Alvarez et al., 2014). On the other hand, emulsions subjected to freezing treatment showed similar degradation among all samples (0.09–0.12%). EPA+DHA degradation was similar in all emulsions and the control, with no significant differences ($p < 0.05$) among them. Although small, the percentage of EPA+DHA quantified in emulsions after freeze-thaw cycling was significantly lower than 100%; this can be attributed to the presence of oxygen in the head-space of the sample containers and the total oil extraction process. Unlike those results obtained at 60 °C, the lack of significant differences among treatments, compared to the surface oil content, could be due to the fact that oxidation of EPA+DHA decreases at lower temperatures. Therefore, the surface oil did not oxidize as fast as those kept at high temperatures.

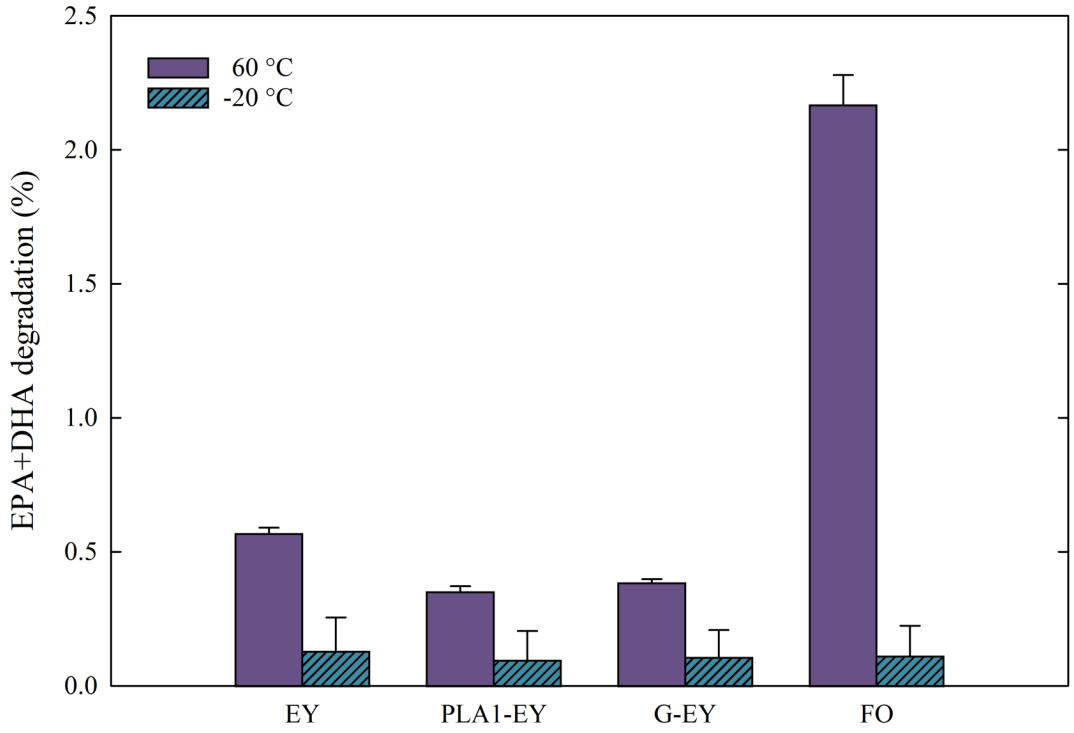

Figure 6.2 Percentage of EPA+DHA degradation in emulsions after 2 hours of incubation at 60 °C or after freeze-thawing cycle. The x axis refers to the treatments egg yolk (EY), phospholipase A_1-treated egg yolk (PLA_1-EY), and egg yolk/gum guar emulsions (G-EY). All treatments were significantly higher than 0. Different letters mean significant differences among treatments under the same conditions. An α= 0.05 was set to assess significant differences.

6.4.2 EPA+DHA degradation during processing and storage of food products

Three emulsions, EY, PLA_1-EY, and G-EY, were incorporated into different food formulations: ice cream and cake. Each food product was selected to represent different processing and storage conditions that are commonly used in the food industry. The efficiency of each carrier was assessed according to their ability to preserve the original content of EPA+DHA (250 mg/90g food product) throughout the food processing. Hereafter, the stage "processing" refers to ice cream base or cake batter; "final product" refers to churned ice cream or baked cake; and the final stage, "Storage" refers to the samples after 10 days of storage at -20 °C or 4–6 °C for ice cream and cake, respectively. The outcomes from these analyses gave an overview of the feasibility of each formulation as delivery system of EPA+DHA in food products.

6.4.2.1 Vanilla ice cream

The factors influencing the content of EPA+DHA in the ice cream were the treatment and the stage of processing ($p < 0.001$); while no significant effect was observed from their interaction. The EPA+DHA content in ice cream at different processing stages are shown in Figure 6.3a. At processing stage or cooking of the ice cream base (86 °C, 3-4 min), a significant reduction ($p < 0.05$) of the initial content of EPA+DHA (250 mg EPA+DHA per 90g, considered as 100%) was observed, with average contents ranging from 243.2–245. 3 (97.3 and 98.1%, respectively) on the cooked base. However, the content in the ice cream base containing non-encapsulated fish oil was significantly lower, losing about 8.77% (21.9 mg) of its original EPA+DHA content. In the next stage, churning of the ice cream, samples showed the same pattern as in processing, being FO samples significantly lower than those containing emulsions, but no significant differences were observed between stages. After 10 days of storage, the ice cream samples showed a significant ($p < 0.001$) degradation of EPA+DHA, compared to their content after processing.

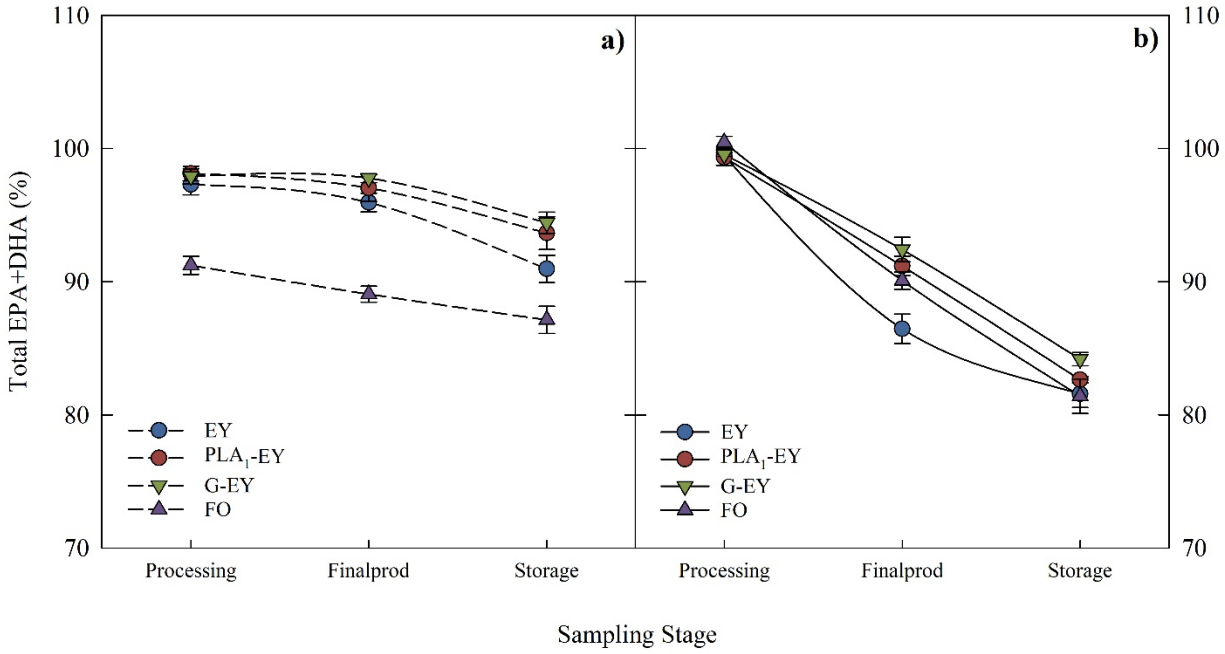

Figure 6.3 Total EPA+DHA content in a) vanilla ice cream and b) vanilla cake, as given by their relative percentage, considering 2.77 mg EPA+DHA/g food product as 100%. The treatments tested were egg yolk (EY), phospholipase A_1-treated egg yolk (PLA_1-EY), egg yolk/gum guar emulsions (G-EY), and non-encapsulated fish oil triglycerides as control (FO). * indicates significantly different within treatments at the same sampling stage. An $\alpha = 0.05$ was set to assess significant differences.

Even though samples were kept under freezing conditions, oxidation had started after cooking of the base; lipid peroxides formed at this first stage could have acted as prooxidants (McClements, 2016), leading to the significant degradation of EPA+DHA during storage. Additionally, it has been shown that under freezing storage (-20 °C), aggregation of egg yolk's low-density lipoproteins occurs, which increases the diameter of such particles thus favoring the contact of the encapsulated material with prooxidants from its surroundings (Martinet, 2003; Meynier et al, 2014).

6.4.2.2 Vanilla cake

The analysis of variance showed a significant influence of treatment ($p < 0.001$), stage of processing ($p < 0.001$), and their interaction ($p < 0.01$) on the total EPA+DHA content of the vanilla cake samples. These effects are shown in Figure 6.3b. All samples taken during processing stage (whisking of the ingredients) showed no significant changes with the original EPA+DHA content. However, the EPA+DHA content reduced significantly in all samples after baking, being EY (86.45%) the only treatment significantly different from the others.

Contrary to what was expected, control samples (FO) did not show significant differences among those containing fish oil from emulsions. This result was due to the fact that, as part of the cake processing, a whisking step is conducted where the yolks, whites, butter, and sugar are mixed at relatively high speeds (as described in section 5.2.5.2). This step caused the fish oil to be incorporated into the whole matrix, resulting in an encapsulation effect. Moreover, unlike ice cream, cake also contains egg white, which have also shown emulsifying properties (McClements, 2016). After 10 days of storage, samples showed significantly lower EPA+DHA content compared to the previous stages, but there were no significant differences among treatments. As seen in Figure 6.3b, the EY treatment was significantly lower than that of the control (FO) after baking. This difference could be due to the processing steps (primary and secondary homogenization) to form the EY emulsions, which caused a minimal deterioration of the EPA+DHA content (data not shown) that together with the extreme temperatures during baking promoted a more rapid development of oxidation of the fatty acids.

6.4.3 Consumer acceptability of the developed *n*-3 fortified food products

One of the main issues related to the fortification of food products with *n*-3 LC-PUFAs is the development of fishy taste. Therefore, the primary focus of this sensory study was to assess consumer acceptance of *n*-3 fortified food products, as given by the liking scores for characteristics such as flavor and aroma.

6.4.3.1 Consumer acceptability of fortified vanilla ice cream

A total of 52 participants, 36 females and 16 males, were recruited for vanilla ice cream tasting. The majority of participants (22 subjects) were 18–25 years old, followed by 20 participants between 26–35 years old. A minority of participants were 36+ years old. As per frequency of consumption, 43% of the subjects declared to consume ice cream at least once a month; while 23% consumed the product once a week. Three formulations and a negative control (using non-encapsulated fish oil) of the same product were evaluated. Differences among formulations consisted on the type of carrier: EY, PLA_1-EY, or G-EY.

Statistical analyses of the data collected indicated a strong treatment effect on the overall liking scores of vanilla ice cream; therefore, the information collected was analyzed from a product perspective (product-specific) rather than consumer. The overall scores suggested the control treatment, FO, as the least liked by consumers. Individual evaluations of flavor, aroma, and overall liking (Figure 6.4) also showed that samples containing FO were the least liked for each descriptor, being the lowest average score of 5.0 for flavor. Although products containing emulsions showed no significant differences in consumer preference among them, the average scores for overall liking and flavor of G-EY ice cream was higher than the other treatments, with 6.9 and 7.1, which positions it in the “like moderately” points of the hedonic scale.

In the option for comments about the least liked sample, the most used descriptions were regarding the aftertaste and flavor, such as “bad flavor”, “fishy taste”, “unpleasant aftertaste”, and “rancid notes”. High concentrations of polyunsaturated fatty acids in food products makes them especially susceptible to oxidation (Jacobsen, 2008). In a first stage, oxidation of lipids is known to cause the formation of hydroperoxides, which can decompose to secondary products such as aldehydes, ketones, acids, and alcohol, causing characteristic off flavors and odors (Jacobsen and Timm, 2001; Lee et al., 2003), commonly refer to as “oxidative rancidity”.

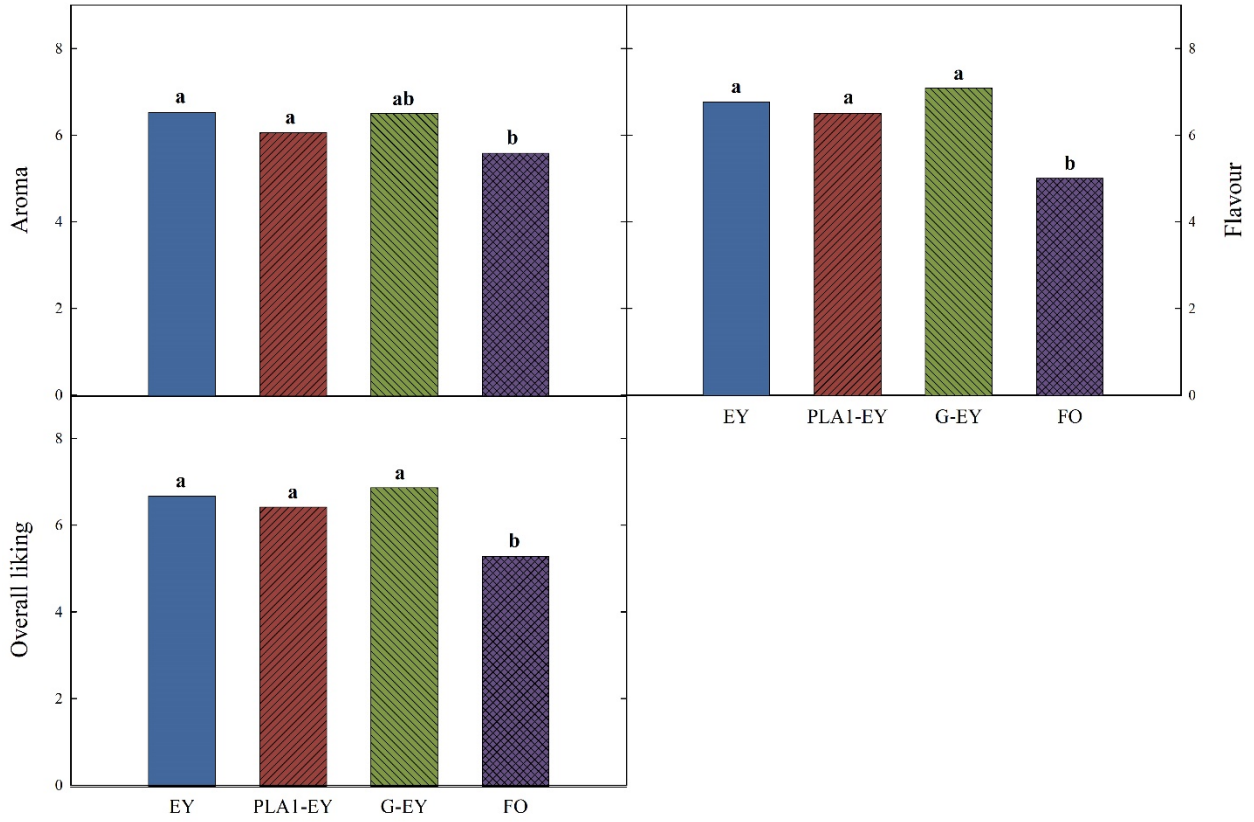

Figure 6.4 Consumer acceptability of vanilla ice cream fortified with 250 mg EPa+DHA/90g product, as given by their aroma, flavor, and overall liking scores, using a 9-point hedonic scale. The x axis refers to the ice cream samples using egg yolk (EY), phospholipase A_1-treated egg yolk (PLA_1-EY), and egg yolk/gum guar emulsions (G-EY) as delivery systems of EPA+DHA, and non-encapsulated fish oil triglycerides (FO) as control. 52 participants were recruited for the test. Different letter means significant differences using Fisher's LSD test with Bonferroni p-value adjustment.

Aldehydes such as (E,E)-2,4-heptadienal and (E,Z)-2,6-nonadienal, have been detected in fish oil and have been associated with off flavors described as oxidized, painty, and green (Karahadian and Lindsay, 1989). The formation of these volatile compounds could have then influenced the consumer's descriptions of the off flavors and aftertaste in the control samples. Moreover, those panelists that liked least samples such as EY and G-EY, noted mainly the texture. Furthermore, some panelists noted "soy-like flavor" or "slight bitterness" in samples containing PLA_1-EY emulsions. These taste notes could be related to the lysophospholipids formed during the enzymatic treatment of egg yolk with phospholipase A_1.

6.4.3.2 Consumer acceptability of fortified vanilla cake

A total of 55 participants, 40 females and 15 males, were recruited for vanilla cake tasting. 23 participants were 18–25 years old, and 23 more between 26–35. A minority of participants were 36+ years old. As per frequency of consumption, 42% of the subjects declared to consume cake at least once a month; while 29% consumed the product once a week. Four treatments, including a control (using non-encapsulated fish oil) were evaluated by each participant.

Statistical analyses indicated a strong panelist effect on the overall liking scores of vanilla cake samples. Thus, the data were analyzed from a consumer perspective (consumer-specific). Nevertheless, after adding the panelist effect to the statistical model, no significant differences were found among product preference (Figure 5.5).

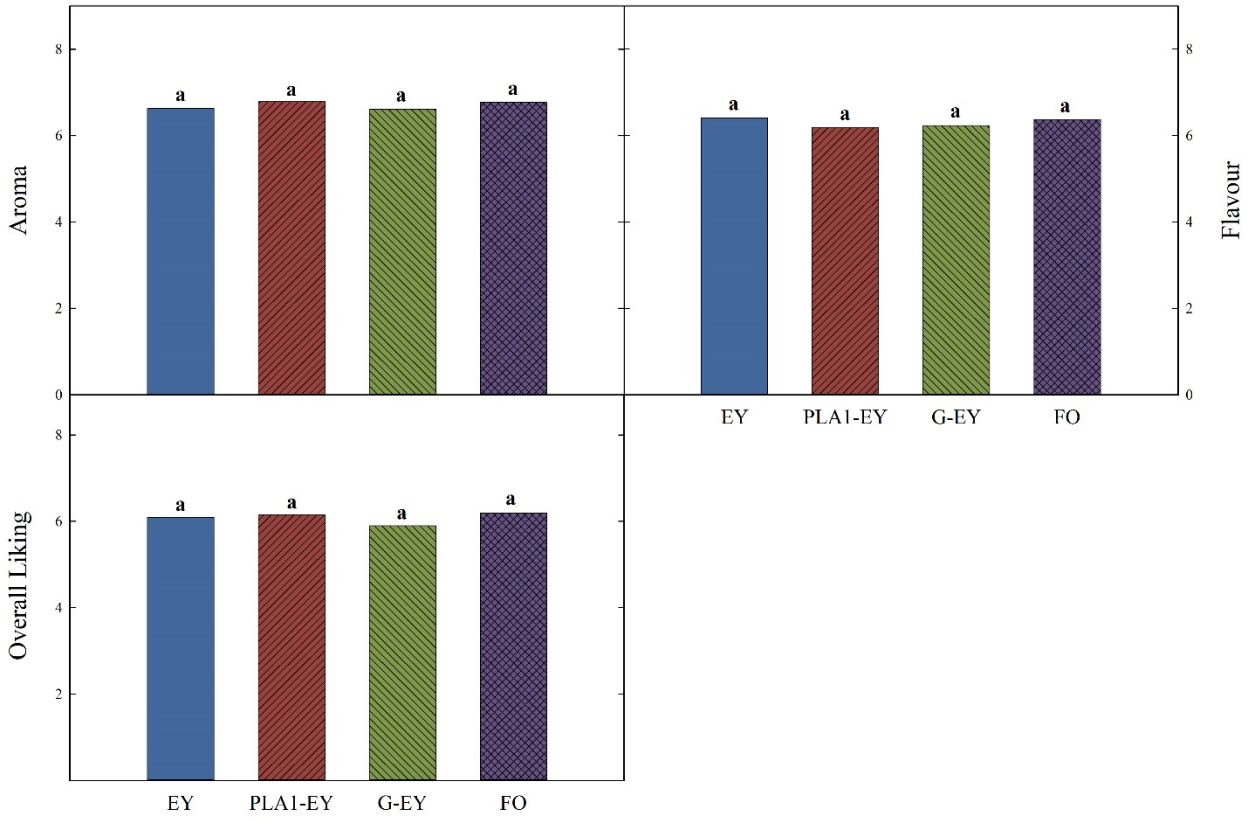

Figure 6.5 Consumer acceptability of vanilla cake fortified with 250 mg EPA+DHA/90g product, as given by their aroma, flavor, and overall liking, scores, using a 9-point hedonic scale. 56 participants were recruited for the test. The x axis refers to the cake samples using egg yolk (EY), phospholipase A_1-treated egg yolk (PLA_1-EY), and egg yolk/gum guar emulsions (G-EY) as delivery systems of EPA+DHA, and non-encapsulated fish oil triglycerides (FO) as control. Different letter means significant differences using Fisher's LSD test with Bonferroni p-value adjustment.

The overall liking showed average scores ranging from 5.9 to 6.2. Although these scores did not fall in the dislike category, they were closer to the neutral point ("neither like nor dislike") than those obtained for ice cream. It was found that the overall liking of cake samples was mostly influenced by the texture and appearance of the products. Descriptors such as flavor and aroma obtained average scores of 6.2–6.4 and 6.3–6.8, respectively, which fell in the "like slightly" scale, and were higher than those found for appearance and texture (5.7 and 5.8, respectively).

In a study conducted by Corey et al. (2015), it was found that consumers did not find significant differences between scrambled egg fortified with *n*-3 LC-PUFAs through hen's feed manipulation and regular table eggs. This lack of significance was attributed to the low content of *n*-3 LC-PUFAs in the final egg product (<45 mg EPA+DHA/50g egg yolk). Nevertheless, in our study, we used about 3 times higher EPA+DHA concentrations (250 mg/90 g food product). Contrary to the panelist comments for ice cream, in the cake study only one subject reported a fishy taste in the control sample. Unlike ice cream, cake samples contained other sources of proteins such as egg white and wheat flour; which could have had a synergistic effect by acting as encapsulating agents (Chee et al., 2005). Additionally, some cake ingredients such as wheat flour are able to form form a matrix that could have prevented the release of volatile components resulting from the oxidation of EPA+DHA, decreasing their perception from the panelists. Therefore, the lack of significant preference among samples was likely due to the effect of the food matrix.

Even though the number of panelists participating in this sensory study (52 for ice cream and 56 for cake) was below our target of 65 panelists, a strong preference for ice cream samples containing egg yolk-based emulsions was found. For cake samples, the lack of significant differences which we attributed to the effect of a more complex food matrix merits further study. This could be done by assessing the volatile compounds release rate which may influence consumer perception.

6.5 Conclusions

This study showed the stability and efficiency of three different egg yolk-based emulsions as delivery systems of EPA+DHA in food products. Ice cream and vanilla cake were selected as model food systems to represent different conditions used during food processing and storage.

The physicochemical analyses showed that egg yolk/gum guar emulsions (G-EY) were the most stable under high temperatures and freeze-thaw cycling as shown by the lack of significant differences in surface oil at both conditions. Moreover, at 60 °C, emulsions were more effective to prevent degradation of EPA+DHA, compared to FO, which had the highest (2.166%). On the other hand, after the freeze-thaw cycling, EPA+DHA degradation was not significantly different among emulsions and the control (0.09–0.12%). A significant preference of ice cream samples containing EPA+DHA over that using non-encapsulated fish oil was found. On the other hand, no significant differences were found in consumer preference of cake samples, which was mostly attributed to the synergistic effect of the emulsions and other ingredients of the food product. The results obtained from this work showed the potential of using egg yolk as a whole food matrix combined with polysaccharides or other food ingredients to protect and prevent EPA+DHA from oxidation and development of off flavors and aromas.

CHAPTER 7: GENERAL DISCUSSION

The information collected and reported throughout this research project supported our hypothesis that egg yolk is an efficient carrier of *n*-3 polyunsaturated fatty acids, such as eicosapentaenoic and docosahexaenoic acids. The literature review indicated that egg yolk presented some of the desired characteristics as a delivery system of lipophilic compounds, such as food-grade, cost-effective production, small particle size, and chemical compatibility with different food matrixes. Other characteristics such as high encapsulation efficiency, and high emulsion and oxidative stability during processing and storage were found in this research.

7.1 General Discussion

During the characterization of egg yolk/fish oil emulsions, we found that the native bimodal particle size distribution of egg yolk was affected by the type of fish oil added. Fish oil triglycerides, but not ethyl esters, increased the proportion of particles in the micro-size fraction resulting in a larger average particle size as given by the Sauter mean diameter (d_{32}). Egg yolk/fish oil emulsions containing 1 and 5% fish oil, regardless the esterification type, showed 100% encapsulation efficiency and negligible EPA+DHA released to the particle surface over 4 weeks of storage at 4 °C. Although EPA+DHA in cooked emulsions remained stable, consumers noticed fishy taste in cooked emulsions containing 5% fish oil. Therefore, the research was then focused on developing a stable emulsion using egg yolk as carrier that could be used as food ingredient.

In the following two studies, we tested higher concentrations of fish oil, ranging from 17 to 50% (w/w on egg yolk dry matter basis), in the emulsions. The effect of processing conditions was also studied, unlike the first study, the following studies tested the inclusion of high-pressure homogenization at 200 MPa on the particle size and stability of the emulsions. High-pressure homogenization resulted in emulsions with better stability under accelerated shelf life conditions, when using raw or phospholipase A_1-treated egg yolk as carriers. On the other hand, it did not significantly affect the stability of the emulsions containing gum guar. This observation could be attributed to the protective effect of gum guar given by its ability to form a network that could embed the particles in the primary egg yolk/fish oil emulsion, rather than the efficiency to form the emulsion. In order to form stable emulsions at higher storage temperature, the egg yolk was

enzymatically modified with phospholipase A_1. Phospholipase A_1-treated egg yolk emulsions showed better stability at 45 °C for up to 10 days of storage and after 2 hours of incubation at 60 °C, compared to those using raw egg yolk. In the assessment of egg yolk/polysaccharide emulsions, gum guar showed improved stability at 45 °C compared to gum arabic. The low efficiency of gum arabic to enhance the stability of egg yolk/fish oil emulsions was attributed to a bridging flocculation effect at the concentrations tested. Furthermore, gum guar formed emulsions with better stability than those using egg yolk or PLA_1-treated egg yolk during freeze-thawing. High-pressure homogenization formed emulsions showed narrower particle size distribution, which had a positive effect on the stability of emulsions using raw and PLA_1-treated egg yolk, but not on the stability of egg yolk/gum guar emulsions. Eicosapentaenoic and docosahexaenoic fatty acids that had been encapsulated in the egg yolk-based emulsions, showed significantly higher oxidative stability than those from non-encapsulated fish oil under different storage conditions.

In addition, the toxicity of the emulsions in Caco-2 cells, regardless the carrier, was significantly lower than that of non-encapsulated fish oil. Finally, the developed emulsions were tested for another important characteristic of delivery systems: it should not adversely alter the quality characteristics of the final food product. For this purpose, a consumer acceptance test was conducted for *n*-3 fortified vanilla ice cream and vanilla cake, using emulsions and non-encapsulated fish oil. A significant preference of ice cream samples containing emulsions over that using non-encapsulated fish oil was found, indicating lower formation of off flavors and unpleasant aromas in the food product. On the other hand, no significant differences were found in consumer's preference of cake samples, which was mostly attributed to the synergistic effect of the emulsions and other ingredients of the food product.

In this research work, we have proven that egg yolk as a whole food matrix can be efficiently fortified with eicosapentaenoic and docosahexaenoic acids using a processing approach. Moreover, egg yolk-based emulsions carrying higher concentrations of *n*-3 fatty acids with enhanced stability can be successfully developed and used as delivery systems in food products. The significance of our results is based on the fact that using egg yolk is easier and more cost-effective than using costly wall materials. Also, the emulsifying property of egg yolk allows to develop emulsions with high stability without the use of surfactants and other

emulsifiers. Therefore, the scope of applications of the developed emulsions can be extended to developing nutraceutical food products that require “clean labels”.

7.2. Future Perspectives

Despite the positive results from this work, the stability and efficiency of the emulsions to carry omega-3 fatty acids could still be improved. Significantly losses of omega-3 fatty acids during processing and storage of the fortified food products, especially after processing that requires high temperature for long time, were observed. Therefore, further work is needed to explore the compatibility of egg yolk with other biopolymers that present stronger electrostatic attraction to the egg yolk proteins to form bilayer emulsions, with the possible inclusion of crosslinking as processing method, that could resist harsher processing conditions. Additionally, the synergistic effect of the formulation ingredients for cake suggests that other food matrices can be used as carriers to encapsulate omega-3 fatty acids. Another aspect that needs to be further researched is the factors affecting the permeability of omega-3 fatty acids through Caco-2:HT29 monolayers and the role of octanoic acid, as well as the relationship between oxidation products and toxicity in Caco-2 cells. Moreover, assessing the bioavailability of the encapsulated eicosapentaenoic and docosahexaenoic acids, using different carriers, during gastrointestinal conditions is an asset to fully support the efficiency of egg yolk-based emulsions as delivery systems.

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