Fractionation and Characterization of Leftover Egg Yolk after Enzymatic Treatment

by

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ABSTRACT

Egg yolk is a rich source of bioactive compounds such as immunoglobulin Y, phospholipids, carotenoids, and amino acids. Supercritical carbon dioxide (SC-CO_2) has been used a green solvent for lipid extraction from egg yolk to a limited extent due to low extraction yield. Cholesterol and phospholipids have been used extensively in pharmaceutical and cosmetic applications. The objectives of this thesis research were to investigate the effects of enzymatic hydrolysis and pellet preparation on the extractability of lipids (neutral lipids, cholesterol and phospholipids) using a two-step SC-CO_2 extraction process and to characterize the fractions obtained. In the first study, egg yolk was treated with a combination of Protease M and Lipase AY-30, which facilitated the formation of a cream layer and a liquid subnatant fraction. Pretreatment of egg yolk could be a viable approach to facilitate destruction of the lipoprotein assembly and to release the lipids. The cream had a higher lipid content (82%) compared to egg yolk (63%). Lipids were extracted in two steps using SC-CO_2 at 48.3 MPa, 70 °C and CO_2 flow rate of 1 L/min (at ambient conditions). In the first step, neutral lipids were extracted using neat CO_2 in which most of the cholesterol was recovered. Polar lipids were extracted in the second step with the addition of 8% (mole%) ethanol as co-solvent. Total extract yield (neutral lipids, cholesterol and phospholipids) from cream was higher than that from egg yolk. The recoveries of cholesterol and PL were 98% and 93%, respectively, based on the feed material used for extraction. Microstructure analysis results showed dissociated individual particles (granules) in the residue samples after SC-CO_2 extraction. The second study focused on the characterization of the potential biological activity such as antioxidant activity of the residue sample after SC-CO_2 extraction. The subnatant phase after hydrolysis and the residue cream sample after SC-CO_2 extraction were rich in small molecular weight peptides. Cellular superoxide generation was detected by dihydroethidium (DHE) to determine the antioxidant activity of egg yolk proteins of the cream residue and the subnatant. The subnatant

showed an antioxidant effect in vascular smooth muscle cells (VSMCs) and endothelial cells against tumor necrosis factor-α (TNF-α) by suppression of the reactive oxygen species (ROS) mediated fluorescence. The findings demonstrate the potential for new opportunities for value-added applications of egg yolk fractions.

PREFACE

This thesis is an original work done by Sule Keskin Ulug and has been written according to the guidelines provided by the Faculty of Graduate Studies and Research at the University of Alberta. The concept of the thesis originated from my supervisors, Dr. Jianping Wu and Dr. Feral Temelli. The research was funded by grants from the Alberta Livestock Meat Agency Ltd., Egg Farmers of Canada, and Natural Sciences and Engineering Research Council of Canada.

The thesis consists of five chapters: Chapter 1 provides a general introduction and the objectives of the thesis; Chapter 2 is a literature review on several subjects, including egg yolk composition and structure, egg yolk lipid extraction by the conventional methods and supercritical CO_2 (SC-CO_2) extraction process, and overall bioactivity and anti-inflammatory activity of hydrolysates derived from egg yolk; Chapter 3 reports the investigation on the effect of enzymatic treatment and pellet preparation by water dilution on the extractability of lipids especially cholesterol, phospholipids and neutral lipids; Chapter 4 evaluates the antioxidant effect of egg yolk hydrolysates obtained after enzymatic treatment by protease and lipase and SC-CO_2 extraction; and the final Chapter 5 provides concluding remarks and future research directions.

Dr. Jianping Wu and Dr. Feral Temelli greatly contributed to the experimental design, data interpretation, thesis preparation and edits. I was responsible for literature review related to the studies above, designing and performing the experiments, data collection, data analysis, and drafting the thesis.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ABTS	2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate)
ACE	Angiotensin converting enzyme
Ang II	Angiotensin II
ANOVA	Analysis of variance
β-CD	β-cyclodextrin
COX-2	Cyclooxygenase-2
DHA	Docosahexaenoic acid
DHE	Dihydroethidium
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DPP-4	Dipeptidyl peptidase-4
DPPH	1,1-diphenyl-2-picryl-hydrazyl
EI	Electron-impact
EPA	Eicosapentaenoic acid
FAME	Fatty acids methyl esters
FBS	Fetal bovine serum
GC-MS	Gas chromatography-mass spectrometry
HDLs	High density lipoproteins
HiM	Helium ion microscopy
IFN-γ	Interferon gamma
IgY	Immunoglobulin Y
IL-17	Interleukin-17

IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	Inducible nitric oxide synthase
LC-ESI-MS-MS	Liquid chromatography-electrospray ionization-Mass Spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDLs	Low density lipoproteins
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPS	Lipopolysaccharide
MFI	Mean fluorescence intensity
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
MUFA	Monounsaturated fatty acids
Neu5Ac/NANA	*N*-Acetylneuraminic acid
NO	Nitric oxide
ORAC-FL	Oxygen radical absorbance capacity-fluorescein
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PGE2	Prostaglandin-E2
PL	Phospholipids
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
RP	Reducing power

RP-HPLC	Reversed phase high-pressure liquid chromatography
SAS	Supercritical antisolvent
SC-CO_2	Supercritical carbon dioxide
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SFA	Saturated fatty acids
SFE	Supercritical fluid extraction
SPE	Solid phase extraction
TBARS	Thiobarbituric acid reactive substances
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor-α
VSMCs	Vascular smooth muscle cells

CHAPTER 1. General introduction and thesis objectives

Eggs are affordable, nutritious and easily accessible, making them an excellent dietary commodity for many people. Egg yolk can be considered as one of nature's perfect foods, containing numerous bioactive components such as lipids, proteins, phospholipids, vitamins, carotenoids among others with health promoting functions (Nimalaratne and Wu 2015). Egg yolk comprises 31% of the weight of fresh whole egg. Fresh egg yolk contains 48% water, 32.9% lipids, 16.4% protein, and 2% carbohydrates (Privett and others 1962). The dry matter of fresh egg yolk varies between 50% and 52% (Anton 2007a)**.**

Egg yolk lipids contain 62% triglycerides, 33% phospholipids (PL), and about 5% cholesterol (Anton 2007a). Egg yolk lipids are mainly composed of lipoproteins. Egg yolk contains 68% LDLs (low-density lipoproteins), 16% HDLs (high-density lipoproteins), 10% livetins, 4% phosvitin and 2% minor proteins on dry matter basis (Anton 2007a). LDLs are the primary lipoproteins in egg yolk and they contain 83-89% lipids and 11-17% protein. LDLs have a complicated structure consisting of complex assemblies of lipids and proteins, which is related to its functional properties such as emulsification. LDLs have a hydrophobic lipid core consisting of triglycerides and cholesterol esters, which is surrounded by a monolayer film, consisting of phospholipids and apoproteins (Cook and Martin 1969; Evans and others 1973).

Egg yolk can be easily fractionated by simple water dilution and centrifugation into pellet and supernatant (Laca and others 2015; Meram and others 2017). The supernatant could be used for immunoglobulin Y (IgY) extraction while that of pellet, containing most lipids (more than 90% of lipids) is referred to leftover egg yolk in the thesis (Kwan and others 1991). Pretreatment of egg yolk by enzymatic hydrolysis could be a viable approach to improve the extraction of lipids, especially phospholipids (Navidghasemizad, Temelli and Wu 2014). Lipases could hydrolyze the lipid core while proteases could hydrolyze apoproteins in the monolayer film surrounding the lipid

core (Navidghasemizad, Temelli and Wu 2014). In the lipid core, lipases can hydrolyze triglycerides and cholesterol esters into free fatty acids and glycerol or cholesterol, which would result in a mixture of unreacted triglycerides, mono- and diglycerides, glycerol, free fatty acids and cholesterol in the lipid core after the enzymatic treatment. Destruction of the assembly of lipoprotein could provide easy access to the supercritical carbon dioxide (SC-CO_2) during the lipid extraction process (Navidghasemizad, Temelli and Wu 2014). For this reason, different combinations of protease and lipase enzymes were tested to hydrolyze the pellet and to produce a cream layer to be used as feed for the SC-CO_2 extraction process (Navidghasemizad, Acero-Lopez, Curtis, Temelli and Wu 2014). Furthermore, enzymatic hydrolysis is a common method to produce bioactive peptides (Aluko 2015). Enzymatic hydrolysis could improve the extractability of lipids while also help to produce biologically active peptides from egg yolk.

Egg yolk PL are well known for their functional properties such as emulsification. PL have three main classes present in egg yolk: phosphatidylcholine (PC), phosphatidylethanolamine (PE) and lysophosphatidylcholine (LPC) (Navidghasemizad, Temelli and Wu 2014). PC is a bioactive component in egg yolk, which accounts for 86% of total egg yolk phospholipids and this amount is three times more than that in soy phospholipids (Anton and others 2006). Choline has been added into infant formulas because it is an essential nutrient for liver function, brain development and cancer prevention (Gutierrez and others 1997). Hence, PC has broad applications in pharmaceutical and nutraceutical products, especially in infant formulas. Cholesterol is one of the important compounds in egg yolk lipids with an average of 186 mg cholesterol per a large egg (USDA National Nutrient Database). Cholesterol is a building block of hormones and vitamins and is needed to build and maintain cell membranes. It is used in shrimp farming and in nutraceutical and pharmaceutical products. According to the 2015 report on the Dietary Guideline

for Americans, the Advisory Committee has removed the recommendation to limit cholesterol intake to 300 mg per day because of lack of evidence showing the relation between cholesterol consumption and blood cholesterol (Dietary Guidelines Advisory Committee 2015). Therefore, cholesterol is no longer a concern of consumption according to this report.

Supercritical CO_2 (SC-CO_2) extraction is considered as a green method to extract lipids from different food matrices such as whole milk powder (Indira and others 2016), squid viscera (Kang and others 2005), and dehydrated beef (Wehling and others 1992). SC-CO_2 is a desirable solvent to extract lipids and bioactive compounds from different sources because it is non-flammable, non-toxic and there is no solvent residue left in the extracts (Temelli 1992). As a non-polar solvent, SC-CO_2 alone is not sufficient to extract PL in food matrices due to the polar nature of PL. Hence, neutral lipids and PL were extracted in two subsequent steps in which ethanol was used as a co-solvent in the second step to extract polar lipids from canola (Temelli 1992; Dunford and Temelli 1995). Egg yolk PL were extracted using the two-step SC-CO_2 extraction process at 48.3 MPa, 70 °C and ethanol as co-solvent by Navidghasemizad, Temelli and Wu (2014) with a high recovery (94% on feed basis) from yolk cream. The studies on cholesterol extraction using SC-CO_2 are slightly older than that of PL. Two-thirds of egg yolk cholesterol could be removed by neat SC-CO_2 at 31-38 MPa and 45-55 °C by Froning and others (1990). However, studies on the SC-CO_2 extraction of egg yolk lipids especially cholesterol are still limited.

Bioactive peptides are protein fragments composed of 2-20 amino acid residues (Martinez-Villaluenga and others 2017). Egg is an essential source of bioactive proteins and peptides (Zambrowicz and others 2014). Chemical methods have been commonly used to assess the antioxidant activity such as oxygen radical absorbance capacity-fluorescein (ORAC-FL) assay (Nimalaratne and others 2015; Liu and others 2015; Huang and others 2012). Antioxidant peptides

derived from egg yolk proteins have been reported (Duan and others 2014; Yousr and Howell 2015; Yoo and others 2017). Egg yolk phosvitin was hydrolyzed by enzymes at different pressures and antioxidant activity was measured in chemical- and cellular-based models (Yoo and others 2017). The antioxidant activity of peptides from egg yolk protein was studied in chemical-based models by Yousr and Howell (2015). The proteolytic digests of phosvitin from egg yolk was also studied for antioxidant activity (Duan and others 2014) using chemical-based assays. However, chemical-based assays lack biological relevance (Jahandideh and others 2015). There is a lack of studies that investigate the potential antioxidant activity of hydrolysates obtained from egg yolk proteins by cell-based studies.

Based on the above, it was hypothesized that lipolytic and proteolytic enzymatic treatment will enhance the extractability of lipids (triglycerides, free fatty acids, phospholipids and cholesterol) from leftover egg yolk by SC-CO_2 extraction while leading to the formation of bioactive peptides with antioxidant activity. Therefore, the overall objectives of this thesis research were:

1. To fractionate the valuable compounds in egg yolk, and
2. To characterize the fractions obtained for potential value-added applications.

The specific objectives of this thesis research were:

1. To examine the effect of enzymatic hydrolysis and pellet preparation on the proximate composition, total extract yield (from yolk, pellet and cream), cholesterol recovery and purity, phospholipid recovery, microstructure, fatty acid profile and sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) profile of leftover egg yolk using the two-step SC-CO_2 extraction process at 48.3 MPa, 70 °C with the addition of 8% (mole%) ethanol as co-solvent in the second step (Chapter 3), and

2. To examine the effect of enzymatic hydrolysis and the two-step SC-CO_2 extraction process at 48.3 MPa, 70 °C with the addition of 8% (mole%) ethanol as co-solvent in the second step on the production of egg yolk proteins and hydrolysates with antioxidant activity using a cell culture study (Chapter 4).

CHAPTER 2. Literature review

2.1. Egg yolk structure, composition and bioactive compounds in egg yolk

Egg yolk provides vital nutrients such as proteins, lipids, vitamins, and minerals. As a natural oil-in-water emulsion, egg yolk is a supramolecular assembly of lipids and proteins, with extensive interactions between proteins and phospholipids (Anton 2013). The efforts to understand the structure and composition of egg yolk could help its further fractionation. In this section, the egg yolk components and their assembly in the structures of LDLs (low density lipoproteins) and HDLs (high density lipoproteins) were reviewed.

2.1.1. Egg yolk composition and bioactive components in egg yolk

Egg yolk comprises 31% of the weight of fresh whole egg. The fresh egg yolk contains 48% water, 32.9% lipids, 16.4% protein, and 2% carbohydrates (Privett and others 1962). The dry matter of fresh egg yolk is between 50% and 52%, which is dependent on the age of laying hen and the preservation time (Anton 2007a). The primary components of egg yolk are lipids. Egg yolk lipids are composed of 62% triglycerides (all in the form of lipoproteins), 33% phospholipids (PL), and less than 5% cholesterol. Carotenoids, giving the egg yolk its yellow-orange color, are present at less than 1%. Proteins can be in free form or bound in the lipoprotein structure (LDLs or HDLs) as apoproteins. Egg yolk contains 68% LDLs, 16% HDLs, 10% livetins (globular protein), 4% phosvitin (phosphoprotein) and 2% minor proteins on dry matter basis (Anton 2007a). HDLs contain 20-25% lipids, which consist of 65% phospholipids, 30% triglycerides and 5% cholesterol (Cook and Martin 1969). LDLs contain 83-89% lipids and 11-17% protein. Their lipids are made up of 74% neutral lipids and 26% phospholipids (Martin and others 1964). Egg yolk is an essential source of bioactive compounds such as phosvitin, Immunoglobulin Y (IgY), sialic acid, phospholipids, omega-3 fatty acids (ω-3), carotenoids, and amino acids. Some egg yolk components have antioxidant properties. These are phospholipids (10%), phosvitin (4%),

carotenoids (<1%), vitamin E (<1%), and aromatic amino acids (<1% on egg yolk dry matter basis) (Nimalaratne and Wu 2015). The egg yolk components and their bioactive properties are summarized below.

In terms of its fatty acid composition, saturated fatty acids account for 30-35% while monounsaturated fatty acids are present at 40-45% and polyunsaturated fatty acids make up 20-25%. The dominant fatty acids are generally oleic (C18:1), palmitic (C16:0) and linoleic (C18:2) acids, accounting for 40-45%, 20-25% and 15-20% of the total fatty acids, respectively (Kuksis 1992). The esterification of fatty acids in triglycerides on the different positions of glycerol backbone generally shows the same trend such that palmitic acid is in position 1, oleic and linoleic acids are in position 2, and oleic, palmitic, and stearic acids are in position 3 (Kuksis 1992). In addition, ω-3 fatty acids are present in phospholipids, especially in phosphatidylcholine (PC). The main ω-3 fatty acids in egg yolk are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) while arachidonic acid (AA) is the major ω-6 fatty acid.

Cholesterol is one of the important compounds of egg yolk lipids with an average content of 186 mg per a large egg (USDA National Nutrient Database). Generally, it accounts for approximately 5% of total lipids. It is found in two different forms, free form or esterified form. Cholesterol in free form makes up 85-90% of the total cholesterol while 10-15% of cholesterol is found in esterified form. Both forms are found in the LDLs structure. Cholesterol esters are present in the LDLs lipid core and are formed by the esterification of cholesterol with different fatty acids, mainly oleic, palmitic, linoleic and stearic acids (Kuksis 1992).

About one-third of egg yolk lipids is made up of PL (Rossi 2007). Egg yolk PL is rich in choline with a high content of phosphatidylcholine (PC; 66-74%). PC in egg yolk is three times more than that in soy phospholipids (Anton and others 2016). Being an essential nutrient for liver

function, brain development and cancer prevention, choline has been incorporated into infant formulas (Gutierrez and others 1997). PC has an amphiphilic character, which has a non-polar part containing ω-3 fatty acids and a polar part containing choline (Anton and others 2016). PC has long chain polyunsaturated fatty acids such as DHA and AA. Egg yolk PC increases the choline level of plasma and brain, causing acetylcholine synthesis and decreasing the risk of Alzheimer disease (Juneja 1997; Anton and others 2006). PL has broad applications in pharmaceutical and nutraceutical products, especially in infant formulas. In addition, phospholipids in egg yolk have emulsifying properties, which can be employed in phospholipid-based emulsions and liposomes in food, cosmetic and pharmaceutical products.

Egg yolk is rich in vitamins such as lipid-soluble vitamins A, D, E and K, which are stored in egg yolk lipids, and water-soluble vitamins, such as B2 (riboflavin) and B9 (folate). In addition, egg yolk is a source of iron and phosphorus (Anton and others 2006).

Carotenoids give egg yolk its yellow-orange color. The main color components of egg yolk are carotene and xanthophylls (lutein, cryptoxanthin, and zeaxanthin). The concentrations of lutein and zeaxanthin are ten times more than those of cryptoxanthin and carotene (Shenstone 1968). Carotene is a compound commonly found in hen feed and it is oxidized to xanthophylls after digestion.

Phosvitin is a phosphoglycoprotein that accounts for 4% of dry egg yolk and 11% of egg yolk proteins. It is one of the most phosphorylated proteins known due to having a high content of phosphorylated serine. Having a high content of phosphorus is responsible for the biological and functional properties of phosvitin, especially its high metal chelating, bone organogenesis and antioxidant properties (Meram and others 2018). Due to its desirable physicochemical properties and biological activities, there is growing interest in phosvitin extraction for industrial applications

(Meram and others 2018). Having a high content of phosphorus provides strong affinity to metal ions, especially iron in egg yolk since 95% of the iron in yolk is bound to phosvitin. At the molecular level, it consists of two polypeptides of 160 kDa (α-phosvitin) and 190 kDa (β-phosvitin) molecular weight and accounts for 80% of the phosphorus in egg yolk proteins. While α-phosvitin has three or four subunits with a molecular weight of 35-40 kDa, β-phosvitin has four or five subunits with a molecular weight of 45 kDa. These sub-units mainly consist of serine (Anton and others 2007).

Livetins, having three kinds α, β, and γ-livetins, are relatively non-homogeneous. The proportion of these three livetins is 2:5:3 (w:w:w), respectively (Bernardi and Cook 1960; Schade and Chacana 2007). All livetins are water-soluble. The main component of γ-livetin is IgY while it is α-2-glycoprotein for β-livetin and that for α-livetin is albumin (Kovacs-Nolan and others 2005). The most important component of the livetin fraction is IgY because it is analogous to a human antibody. IgY is important for its antibacterial and antiviral properties (Meram and Wu 2017). IgY can be easily separated by the widely used 10-fold dilution method (Kwan and others 1991). IgY is a potential source for immunological applications such as infant formulas.

Another bioactive compound found in egg yolk is sialic acid, which is a monosaccharide with a nine carbon backbone, exhibiting anti-inflammatory activity (Gorog and Kovacs 1978). The most common form of sialic acid is *N*-acetylneuraminic acid (Neu5Ac or NANA).

2.1.2. Egg yolk structure (LDL and HDL structures)

Lipids in egg yolk are linked with proteins in the forms of low-density lipoproteins (LDLs) and high-density lipoproteins (HDLs). Characterization of the interactions between proteins and lipids is crucial to understand the egg yolk structure. Micro- and nano- structures of egg yolk were reviewed by Anton (2013). Egg yolk can be separated into two main fractions (plasma and

granules) using the water dilution method (McBee and Cotterill 1979). Plasma contains mainly LDLs and soluble proteins (livetins) while the granules consist of mainly HDLs and phosvitin bonded by phosphocalcic bridges. Plasma contains 78% of egg yolk dry matter, comprised of 85% LDLs and 15% livetins, while granules contain 22% of egg yolk dry matter, comprised of 70% HDLs, 16% phosvitin and 12% LDLs (Burley and Cook 1961; Saari and others 1964; Anton 2013). To understand the egg yolk structure better, LDL and HDL structures should be clearly analyzed.

LDLs (also known as lipovitellenin) are the main components of egg yolk, which make up 2/3 of total egg yolk dry matter. A large proportion of LDLs is present in plasma, but a small proportion of LDLs is also found in granules (Anton 2007b). LDLs are spherical particles with a diameter between 17 and 60 nm, which have a lipid core consisting of triglycerides and cholesterol esters. This hydrophobic lipid core is surrounded by a monolayer film, consisting of phospholipids and apoproteins (Cook and Martin 1969; Evans and others 1973). LDLs are dispersed in aqueous solutions because of having a low density (0.982 g/mL) (Anton 2007b). Phospholipids play an important role for the stability of LDL structure due to the hydrophobic interactions (Burley 1975). This monofilm also contains some cholesterol, which provides rigidity to the LDL structure (Anton 2007b). LDLs consist of two sub-units: LDL_1 and LDL_2. LDL_1 contains twice as much protein as in LDL_2 while LDL_2 comprises 80% of the total amount of LDLs. Although the chemical composition is similar for both sub-units, the percentages of lipids and apoproteins are different, resulting in their different sizes (Martin and others 1964; Anton 2007b). LDLs contain six major apoproteins and the main apoprotein with a molecular weight of 130 kDa makes up 70% of the total apoproteins (Anton 2007b). The second major apoprotein has a molecular weight of 15 kDa. Apoproteins of LDLs are also glycosylated by hexose, hexosamine and sialic acid (Nakamura and others 1977).

HDLs (also known as lipovitellin) are the second group of lipoproteins, which make up 1/6th of total egg yolk dry matter and 36% of egg yolk proteins (Anton 2007c). HDLs have a molecular weight of 400 kDa with a diameter between 7 and 20 nm (Burley and Cook 1961). HDLs have a globular protein structure as opposed to the LDLs' spherical micelle structure (Anton 2007c). They are mainly proteins, showing more of a resemblance to lipid transfer proteins. HDLs consist of a dimer of monomers with a molecular weight of 200 kDa, which are globular proteins with a funnel-like cavity, containing two β-sheets with mainly hydrophobic amino acids (Anderson and others 1998). Phospholipids surround this hydrophobic cavity, interacting with hydrophobic amino acids (Banaszak and others 1990; Timmins and others 1992). HDLs contain five apoproteins with a molecular weight between 35 and 110 kDa. Apoproteins in HDLs are glycosylated by mannose, galactose, glucosamine and sialic acid and the total amount of carbohydrate is 0.75% (Anton 2007c). HDLs form a complex with phosvitin to form a granule structure (Anton 2007c).

Regarding the granule structure, granules consist of spherical structures with a diameter between 0.3 and 2 μm. This structure mainly consists of insoluble, stable and compact structure of HDLs and phosvitin linked by phosphocalcic bridges (Chang and others 1977; Anton 2013). HDLs have a higher density (1.120 g/mL) compared to that of LDLs (0.982 g/mL), which is close to the density of protein, because they contain 75-80% proteins and 20-25% lipids (Cook and Martin 1969).

2.2. Fractionation of egg yolk components

2.2.1. Separation of egg yolk

The presence of various valuable components in egg yolk, such as IgY, phospholipids and phosvitin, has been receiving growing attention to extract them at commercial level. In addition, using egg yolk efficiently is important from the standpoint of the fractionation, extraction and

complete utilization of the bioactive components and by-products from egg yolk (Meram and others 2018). Egg yolk can be separated easily into pellet (granule) and supernatant (plasma) by dilution and centrifugation although further separation of the egg yolk components is more challenging (Laca and others 2015). IgY (soluble proteins) has been separated from egg yolk using 10 times dilution method in many studies. In our group, Meram and others (2017) separated IgY from egg yolk by 10 times dilution at pH 5 and centrifugation at 2,800xg and 4 °C for 30 min. Secondly, Navidghasemisad, Temelli and Wu (2014) extracted phospholipids from leftover egg yolk after IgY extraction. IgY was separated from egg yolk by 10 times dilution with water at pH 6 and centrifugation at 10,000xg and 4 °C for 15 min by following Kwan and others (1991). Most lipids (above 90% of lipids) was recovered in the pellet (granule) fraction after IgY extraction. On the other hand, the leftover egg yolk (pellet) still had HDLs and LDLs, which are rich in neutral lipids, cholesterol and phospholipids and should be further examined for the extraction of these individual classes of components.

Pretreatment of the pellet with enzymes may enhance the separation efficiency of lipids. Indeed, hydrolysis of the pellet with a combination of protease and lipase enzymes resulted in the formation of a cream fraction after centrifugation, which was a suitable feed material for supercritical carbon dioxide (SC-CO_2) extraction of neutral lipids and phospholipids (Navidghasemizad, Acero-Lopez, Curtis, Temelli and Wu 2014). Different combinations of proteases with Lipase AY30 were used to weaken the pellet emulsion and to release lipids from the pellet, because egg yolk is a natural and stable oil-in-water emulsion. This approach improved the extractability of PL from egg yolk using SC-CO_2 by providing easy access to SC-CO_2 (Navidghasemizad, Temelli and Wu 2014). Hydrolyzing the egg yolk proteins, especially apoproteins by the protease and hydrolyzing the triglyceride core of lipoproteins by the lipase

assisted the extraction of PL. This finding also highlights the potential for the extraction of other components such as cholesterol, but this aspect has not been reported previously.

2.2.2. Egg yolk lipid extraction methods

2.2.2.1. Conventional methods for lipid extraction from egg yolk

Total lipid extraction has been limited to organic solvent extraction for analytical scale applications such as Bligh and Dyer method and Folch method using a mixture of chloroform/methanol or hexane/isopropanol, respectively (Bligh and Dyer 1959; Hara and Hadin 1978; Boselli and Caboni 2000). For large scale of extraction of lipids, the extraction of different classes of neutral lipids, phospholipids, and cholesterol from egg yolk has not been clearly defined. Organic solvents have been used such as hexane, acetone, and dimethyl ether to extract total lipids from egg yolk. Palacios and Wang (2005) developed a method for the extraction of lecithin (PL) from fresh egg yolk by using ethanol. In this study, hexane was used to extract the total lipids, especially neutral lipids from egg yolk and acetone was used for the removal of cholesterol (Palacios and Wang 2005). More recent studies have focused on using less harmful organic solvents to extract lipids from foods such as ethanol. Fractionation of lipids (triglycerides, phospholipids and cholesterol) from fresh egg yolk was recently developed by Su and others (2015) using only ethanol as an organic solvent for the extraction. Egg yolk and ethanol were mixed and heated at 65 °C for 1 h to separate yolk proteins. The ethanol extracted fraction was then subjected to low temperature crystallization at 4 °C. The solidified triacylglycerols were removed by refrigerated centrifugation. The supernatant containing cholesterol and phospholipids was mixed with β-cyclodextrin (β-CD) and then subjected to centrifugation. The precipitate and supernatant of centrifugation were collected for cholesterol determination. The supernatant obtained after cholesterol removal was dried by rotary evaporation to recover phospholipids.

Despite all this effort, using organic solvents for the fractionation of egg yolk by the conventional methods is not sufficient to meet the growing consumer demands for 'natural' and healthy foods.

2.2.2.2. Supercritical CO_2 extraction of egg yolk

Lipid extraction from various matrices has been performed by conventional solvent extraction or by the relatively newer technologies such as supercritical fluid extraction. Today's consumer tends to consume greener products, which encourages the companies to use greener technologies. SC-CO_2 is a desirable solvent to extract lipids and bioactive compounds from different sources because it is non-flammable, non-toxic and there is no solvent residue left in the extracts (Temelli 1992, 2009). SC-CO_2 is a dense fluid, which is kept at temperature and pressure conditions above its critical point of 31.1 °C and 7.4 MPa. Small changes in temperature and pressure in the supercritical region result in changes in the density of CO_2, which allows adjusting the physicochemical properties of the supercritical fluid. While a supercritical fluid has liquid-like density, it also has gas-like diffusivity and viscosity, which contributes to high mass transfer rates. SC-CO_2 is suitable for use instead of organic solvents for both industrial and laboratory uses. Besides, the critical point of CO_2 is easily accessible, allowing the extraction of heat sensitive compounds at mild conditions. However, CO_2 is classified as a non-polar solvent, which is suitable for non-polar solutes and a polar co-solvent is needed if the polar solutes are the target.

The extraction of cholesterol using SC-CO_2 from different sources was studied such as *Eichhornia crassipes* biomass (Martins and others 2016), whole milk powder (Indira and others 2016), squid viscera (Kang and others 2005), and dehydrated beef (Wehling and others 1992). Solubility of cholesterol and its esters in SC-CO_2 was determined at temperatures of 40-80 °C and pressures of 15-25 MPa by Wong and Johnston (1986), Yun and others (1991), Kosal and others (1992). However, inconsistency in the solubility data generated by using different methods makes

the comparison difficult. As expected, an increase in pressure resulted in an increase in cholesterol solubility at constant temperature; however, use of a combination of high temperature and pressure has not been reported for extraction purposes (Froning and others 1990; Warren and others 1991; Sun and others 1995).

The extraction of PL from different sources using SC-CO_2 + ethanol was reported recently such as pacific saury (Zhang and others 2018), Atlantic salmon by-product (Haq and Chun 2018), buttermilk powder (Barry and others 2017), and scallop by-product (Subra-Paternault and others 2015). A laboratory scale supercritical fluid extractor was used to extract phospholipids from canola by Dunford and Temelli (1995). Navidghasemizad, Temelli and Wu (2014) studied the extractability of egg yolk phospholipids at 48.3 MPa, 70 °C and CO_2 flow rate of 1 L/min at ambient conditions. The conditions were chosen to maximize the solubility of lipids based on the solubility data of fatty acids, mono-, di-, and triglycerides in SC-CO_2 as reviewed by Guclu-Ustundag and Temelli (2000). In addition, ethanol was added as a co-solvent based on the data reported by Guclu-Ustundag and Temelli (2005) to extract polar lipids from egg yolk.

2.2.2.2.1. Cholesterol extraction by SC-CO_2 from egg yolk

Limited studies were conducted on the extraction of cholesterol from egg yolk using SC-CO_2. Previous studies were focused on the removal of cholesterol from egg yolk rather than its extraction for further value-added applications (Froning and others 1990; Warren and others 1991; Sun and others 1995). In addition, detailed analyses, including cholesterol extraction kinetics have not been reported. The motivation for cholesterol removal in the earlier studies was the previously claimed health risk of dietary cholesterol to increase the risk of heart disease while this view has been recently revoked (Dietary Guidelines Advisory Committee 2015; Ravnskov and others 2018). The above studies were in agreement on the extraction of cholesterol using neat SC-CO_2 due to

the non-polar nature of cholesterol. An increase in temperature and pressure of SC-CO_2 simultaneously could result in a decrease in the content of lipids and cholesterol to produce a low-fat and low-cholesterol egg product for the industry. Spray-dried egg yolk was used as the feed material in most studies but different fractions of egg yolk such as cream or pellet have not been investigated. The removal of cholesterol and lipids under mild conditions resulted in increased concentrations of protein and polar lipids (phospholipids) in the egg yolk residue samples. Another aspect evaluated was to remove the cholesterol from egg yolk while maintaining the functional properties of phospholipids in the egg yolk residue (Froning and others 1990).

The fundamental studies on cholesterol extraction using SC-CO_2 were carried out by Froning and others (1990), Sun and others (1995) and Warren and others (1991). Froning and others (1990) removed cholesterol from dried egg yolk using neat CO_2 at different operating conditions (16.5 MPa/40 °C, 24.1 MPa/45 °C, 31.0 MPa/45 °C, 37.9 MPa/55 °C) without the removal of polar lipids. They showed that an increase in temperature and pressure enhanced the removal of cholesterol and neutral lipids. The high temperature and pressure (37.9 MPa/55 °C) conditions resulted in the removal of two-thirds of cholesterol from egg yolk and an increase of phospholipid content in the egg yolk residue. However, higher levels of temperature and pressure were not examined even though they realized an increase in these parameters promoted the removal of cholesterol. In addition, varying temperature and pressure simultaneously makes it difficult to understand the effects of pressure or temperature alone. Sun and others (1995) investigated the effects of extraction temperature (40-60 °C) and pressure (15-35 MPa) on the SC-CO_2 extraction of the total lipids and cholesterol from dried egg yolk. Warren and others (1991) also followed the conditions employed by Froning and others (1990) to extract cholesterol from egg yolk without removing polar lipids to maintain their functional properties. They also compared the recovery of

SC-CO_2 extraction to that of the conventional hexane extraction. The highest cholesterol reduction (98%) was observed at 37.9 MPa and 55 °C using SC-CO_2, which was substantially higher than that for hexane extraction (37%).

Boselli and others (2001) extracted cholesterol at 33.8 MPa and 40 °C from egg containing food products (biscuits, noodle and snack) and purified the cholesterol using solid phase extraction (SPE). The results were compared with the conventional method (Soxhlet-SPE), showing that the two methods gave similar results, demonstrating the potential use of green technology as an alternative. After lipid extraction, SPE was performed to separate the non-polar lipids and to purify the cholesterol prior to gas chromatography analysis (Boselli and others, 1997). Later, Boselli and others (2002) determined the cholesterol oxidation products in dried egg yolk using neat SC-CO_2 at 51.7 MPa and 40 °C. The results were compared with two conventional lipid extraction methods (Bligh and Dyer method and Folch method), where the recoveries from supercritical extraction and the conventional methods were similar.

The effect of different moisture levels (dried, 7% and 12%) of dried egg yolk on the efficiency of cholesterol and lipid extraction using SC-CO_2 was only studied by Froning and others (1998) at the previously determined optimum conditions of 31 MPa and 45 °C. Increasing the moisture content to 7% and 12% did not affect the extraction of cholesterol compared to the control. The presence of 7% moisture in dry egg yolk was recommended for SC-CO_2 extraction of lipids; however, a higher amount of water reduced the extractability of lipids.

Additional studies were conducted to determine the functional properties of cholesterol-reduced egg yolk obtained by using SC-CO_2 in oil-in-water emulsions (Bringe and others, 1996; Paraskevopoulou, Panayiotou and Kiosseoglou, 1997; Paraskevopoulou and Kiosseoglou, 1997; Paraskevopoulou and others 1999). The cholesterol content of the spray-dried egg yolk was

reduced using SC-CO_2 at 31.4 MPa and 35 °C by Paraskevopoulou and others (1999) and the rheological properties of cholesterol-reduced egg yolk were examined in a mayonnaise-like emulsion. SC-CO_2 extraction was found to be more efficient resulting in samples with lower cholesterol and higher phospholipid contents compared to that of solvent extraction. Paraskevopoulou, Panayiotou and Kiosseoglou (1997) extended the study of Froning and others (1990) to understand the functional properties after the removal of cholesterol. The majority of cholesterol (75% of yolk cholesterol) was removed by SC-CO_2 regardless of the extraction conditions. Higher foam activity and foam stability were achieved using the cholesterol-reduced yolk by SC-CO_2 extraction compared to those of control. On the other hand, Paraskevopoulou and Kiosseoglou (1997) compared the recoveries of SC-CO_2 and solvent extractions. Both methods achieved the removal of cholesterol and lipids while enhancing the concentrations of protein and polar lipids in the residue. The high protein content of the extraction residue resulted in stronger and more elastic structures compared to that of commercial mayonnaises. SC-CO_2 extraction removed 77% of total cholesterol from egg yolk while it was 73% for petroleum ether extraction (Paraskevopoulou and Kiosseoglou 1997).

In conclusion, the studies reported to date showed that there is a need for cholesterol extraction from egg yolk with high recovery and purity. Most of the previous studies focused on collecting one extract at the end of the extraction process and analyzed the cholesterol content of the residue sample. It is important to understand the extraction kinetics of cholesterol to potentially design further fractionation approaches. Besides, recent developments have led to new applications of cholesterol such as in shrimp farming, and cosmetic and pharmaceutical products. Therefore, cholesterol extracted from egg yolk may have potential new uses.

2.2.2.2.2. Phospholipid extraction by SC-CO_2 from egg yolk

Table 2.1 summarizes the studies that reported the extraction of lipids from egg yolk. Several studies reported PL extraction from dried egg yolk using SC-CO_2 (Shah and others 2004; Aro and others 2009; Navidghasemizad, Temelli and Wu 2014). In these studies, polar lipids were extracted with the addition of ethanol into SC-CO_2 as co-solvent to enhance the polarity of SC-CO_2. Shah and others (2004) optimized the supercritical fluid extraction conditions to extract the neutral lipids in the first step and ethanol was used in the second step to extract PL from dried egg yolk. The highest yield of PC was reported as 49 g PC/kg of dried egg yolk under optimum conditions (41.4 MPa, 60 °C, CO_2 flow rate of 5 L/min measured at 0.1 MPa and 25 °C with 0.5 molar fraction of ethanol). Previously, Caboni (2000) extracted the total lipids, including PL, using SC-CO_2 without the addition of organic co-solvent from dried egg yolk at 51.7 MPa and 40 °C. The results were compared with that of the conventional Bligh and Dyer method. SC-CO_2 extraction method was considered as a viable alternative giving similar results with 67 g extract/100 g sample as compared to 63 g extract/100 g sample obtained with the conventional method. The neat SC-CO_2 extract contained 26% PL while the solvent extract contained 29% PL. However, detailed information was not provided in terms how PL was extracted using neat SC-CO_2 without any co-solvent addition. Later, Aro and others (2009) used similar operating conditions (45 MPa, 70 °C) and ethanol as co-solvent. SC-CO_2 + ethanol extraction was used for the isolation of high purity yolk phospholipids and supercritical anti-solvent (SAS) process was used to precipitate phospholipids from egg yolk (powder and granule). Neat CO_2 was used to deoil the sample in the first step, followed by SC-CO_2 + ethanol to extract phospholipids in the second step. Then, SAS process was employed after the second extraction step to increase the purity, which resulted in high purity PL (72-99%) depending on the process conditions.

Table 2.1. Summary of published studies based on SC-CO_2 extraction of lipids from egg yolk

Material used	Target compound	Operating conditions	Key findings	Reference
Spray-dried egg yolk	Cholesterol	16.5 MPa/40 °C, 24.1 MPa/45 °C, 31.0 MPa/45 °C, 38.0 MPa/55 °C	Protein and phospholipids were concentrated by SC-CO_2 extraction. Extraction at 31 MPa/45°C and 38 MPa/55°C removed two-thirds of the total cholesterol.	Froning and others (1990)
Spray-dried egg yolk	Cholesterol	24.1 MPa/45 °C, 31.0 MPa/45 °C, 38.0 MPa/55 °C	Residual total lipid and cholesterol contents decreased at the higher pressure and temperature.	Warren and others (1991)
Egg containing food (biscuits, noodle and snack)	Cholesterol	33.8 MPa/40 °C	Cholesterol was extracted and purified by on-line SC-CO_2-SPE. Free cholesterol extraction results similar to that of Soxhlet extraction.	Boselli and others (2001)
Dried egg yolk, pellet and cream	Phospholipids	48.3 MPa/70 °C Ethanol as co-solvent	The average recovery and purity of PC from the cream were 94% and 57% compared to 47% and 47%, and 40% and 75% for dry yolk and pellet, respectively.	Navidghasemizad, Temelli and Wu (2014)
Egg yolk powder and granules	Phospholipids	48.3 MPa/70 °C Ethanol as co-solvent	The recovery for phospholipid extraction was 40-50% with a purity of 72-99% using SC-CO_2 + SAS.	Aro and others (2009)

*SPE: Solid phase extraction, SAS: Supercritical antisolvent

Low recoveries were achieved in most studies up to the recent study of Navidghasemizad, Temelli and Wu (2014). Pretreatment of egg yolk by enzymatic hydrolysis was done to degrade the LDL structure and to release PL, which are attached to apoproteins. They reported that the recovery and purity of PC from the cream were 94% and 57% compared to 47% and 47% for dried egg yolk.

Although the moisture content of the feed material is very important for potential scale up of the supercritical extraction method, the effect of moisture content on the PL extraction was not studied extensively. Navidghasemizad and others (2015) studied the effect of different moisture levels in cream samples (freeze dried, 20% and 45% of moisture) and egg yolk samples (fresh and dry egg yolk) on the extractability of phospholipids using SC-CO_2. Cream with 20% moisture showed a high recovery of PC (106%) and PE (44%). The results showed that moisture contents of up to 20% were favorable for the extractability of PL and PC from egg yolk.

In conclusion, achieving high PL recovery using SC-CO_2 extraction is still a relevant challenge and more studies are needed to achieve the maximum recovery and purity of the different types of PL.

2.3. Bioactivity of egg yolk protein hydrolysates

Bioactive peptides are specific protein fragments that are encrypted in proteins. These peptides can only show their biological effects once they are released from their parent proteins. Biologically active proteins usually contain 2-20 amino acid residues (Korhonen and Pihlanto 2006). Hence, production of hydrolysates from the parent protein by conventional or novel technologies mentioned above is the initial step for the recovery of bioactive peptides, which requires subsequent purification steps. There are several reports on the identification and characterization of bioactive peptides or hydrolysates from egg yolk proteins focused mainly on

their antioxidant, anti-inflammatory, angiotensin converting enzyme (ACE) inhibitory or anti-hypertensive, and anti-diabetic properties. A summary of some studies reporting on the bioactive hydrolysates from egg yolk proteins is presented in Table 2.2.

Yousr and Howell (2015) investigated the ACE-inhibitory activity by fractionation and purification of the isolated peptides. Third fraction (EYGF-56) showed high ACE-inhibitory activity (about 69%). It was concluded that leucine (binding Zn^{2+}), positively charged lysine and arginine and hydrophobic tryptophan in the peptide sequence of EYGF-56 fraction could be responsible for the ACE-inhibitory activity. You and Wu (2011) reported that the egg protein (egg white and egg yolk) hydrolysates produced by non-gastrointestinal enzymes (thermolysin and alcalase) presented stronger ACE-inhibitory activity and a high proportion of positively charged amino acids made a major contribution. Majumder and Wu (2009) studied the effect of different cooking methods (fried or boiled eggs) on the egg protein peptides (egg white and egg yolk) with ACE-inhibitory activity. Gastrointestinal digestion was performed under simulated gut conditions. Fried egg samples showed a higher potential activity. Seven peptides were characterized by liquid chromatography-tandem mass spectrometry (LC-MS/MS). They reported that *in vivo* digestion of cooked egg helped release of potent peptides from egg, demonstrating their potential use in various applications, targeting cardiovascular disease prevention. Eckert and others (2014) aimed to study the ACE-inhibitory activity of hydrolysates prepared from egg yolk protein after phospholipid extraction and to purify and confirm the activity of peptides derived from egg yolk hydrolysates. After determining the ACE-inhibitory activity of the hydrolysates, peptides showing ACE-inhibitory activity were purified from the hydrolysates. Those peptides had 9-18 amino acids with arginine and leucine at the N-terminal position. Peptide with the LAPSLPGKPKPD sequence presented the strongest ACE-inhibitory activity.

Table 2.2. Summary of some studies focusing on bioactive hydrolysates from egg yolk protein.

Hydrolysate source	Bioactivity	Key findings	Reference
Egg yolk residue after PL extraction	Antioxidant activity	Two different fractions (EYGF-23) and (EYGF-33) showed antioxidant activity. Higher amount of hydrophobic amino acids in the sequence of these fractions correlated to higher antioxidant activity.	Yousr and Howell (2015)
Egg yolk residue after PL extraction	Antioxidant, ACE-inhibitory and anti-diabetic activities	YIEAVNKVSPRAGQF had ACE-inhibitory activity, YINQMPQKSRE had the highest antioxidant activity and DPP-4 inhibitory activity and VTGRFAGHPAAQ peptide showed anti-diabetic properties to α-glucosidase.	Zambrowicz and others (2015a)
Egg protein (egg yolk and egg white)	Antioxidant and ACE-inhibitory activities	Protein hydrolysates produced by thermolysin and alcalase showed a lower antioxidant activity compared to those produced by gastrointestinal enzymes. Hydrolysates produced by non-gastrointestinal enzymes had stronger ACE-inhibitory activity and a high proportion of positively charged amino acids.	You and Wu (2011)
Defatted egg yolk protein	Antioxidant activity	Hydrolysates showed the strongest antioxidant activity against lipid oxidation compared to the egg yolk protein and amino acid mixture of egg yolk.	Sakanaka and others (2004)
Egg yolk residue after PL extraction	Antidiabetic activity (α-glucosidase and DPP-4 inhibitory activities)	LAPSLPGKPKPD had the strongest α-glucosidase and DPP-4 inhibitory activities.	Zambrowicz and others (2015b)
Egg yolk phosvitin	Antioxidant and anti-inflammatory activities	Hydrolysates prepared at 100 MPa showed higher reducing power and free radical scavenging activity compared to those obtained at atmospheric pressure.	Yoo and others (2017)

*DPP-4: Dipeptidyl peptidase-4

Anti-diabetic activities of egg yolk protein hydrolysates were also reported in some studies (Zambrowicz and others 2015a and 2015b). The anti-diabetic properties such as α-glucosidase and dipeptidyl peptidase-4 (DPP-4) inhibitory activities were examined using the peptides derived from egg yolk protein residue after phospholipid extraction (Zambrowicz and others 2015b). The peptide with the sequence LAPSLPGKPKPD presented the strongest α-glucosidase and DPP-4 inhibitory activities.

Phosvitin, which is a highly phosphorylated egg yolk storage protein, is reported to have metal-chelating, antioxidant and emulsifying activities (Yu and others 2014). Phosvitin was hydrolyzed by different proteases at different pressures to produce phosvitin hydrolysates by Yoo and others (2017). This study determined the effect of high hydrostatic pressure and enzymatic hydrolysis on antioxidant and anti-inflammatory properties of phosvitin hydrolysates. Hydrolysates produced at 100 MPa showed higher antioxidant properties compared to those obtained at ambient conditions. The hydrolysate showing the highest antioxidant and anti-inflammatory activity was analyzed by LC-MS/MS to determine its sequence.

In conclusion, egg yolk has a high potential for the generation of bioactive peptides; however, larger scale production of peptides is needed to generate sufficient quantities to be able to carry out clinical studies. Biologically relevant studies especially cell-based assays should be executed for better understanding of their activities. In addition, the number of studies is still limited for egg yolk protein hydrolysates compared to other hydrolysates derived from animal or plant sources.

2.3.1. Antioxidant activity of egg yolk hydrolysates

Egg proteins have been used extensively to produce antioxidant peptides by enzymatic hydrolysis. Egg yolk has antioxidant compounds such as lutein, zeaxanthin, phosvitin and free amino acids (Nimalaratne and others 2011; Nimalaratne and Wu 2015). Thermal processing could

cause loss of antioxidant properties in eggs while gastrointestinal digestion could increase the antioxidant properties in eggs due to the formation of free amino acids and peptides (Nimalaratne and Wu 2015). However, the antioxidant activity of egg yolk has been studied only to a limited extent, especially using cell-based studies. The public perception on high content of cholesterol in egg yolk may have had a negative effect (Nimalaratne and others 2011); however, there is no direct link between blood cholesterol levels and egg yolk consumption (Lee and Griffin 2006; Qureshi and others 2007; Nimalaratne and others 2011).

Antioxidant activity is commonly studied using chemical assays such as oxygen radical absorbance capacity-fluorescein (ORAC-FL) assay, ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate)) radical anion scavenging activity, DPPH (1,1-diphenyl-2-picryl-hydrazyl) radical scavenging activity and reducing power (RP) (Nimalaratne and others 2015; Liu and others 2015; Wang and others 2018). Egg yolk components such as digests of phosvitin showed a strong antioxidant activity by chemical assays (Xu and others 2007). Nimalaratne and others (2011) also characterized the phenolic compounds in egg yolk with antioxidant properties by the ORAC, DPPH and ABTS assays.

There are several studies focusing on the antioxidant activity of peptides or hydrolysates isolated from egg yolk; some with more detail on their amino acid sequence (Yousr and Howell 2015; Zambrowicz and others 2015). Yousr and Howell (2015) produced peptides by enzymatic treatment with pepsin and pancreatin from the egg yolk residue after PL extraction. They fractionated and purified the egg yolk hydrolysate by ultrafiltration and gel filtration to produce gel-filtered fractions (EYGF). Two promising fractions (EYGF-23) and (EYGF-33) showed radical scavenging and metal chelating ability as well as inhibition of peroxides and thiobarbituric acid reactive substances (TBARS). Higher amount of hydrophobic amino acids in the sequence of

these fractions corresponded to a higher level of antioxidant activity. Zambrowicz and others (2015) examined the peptides with antioxidant and ACE-inhibitory activities and anti-diabetic properties with α-glucosidase and DPP-4 inhibitory activities from egg yolk protein residue after PL extraction. The peptides were fractionated and purified by ion-exchange chromatography and reversed phase high-pressure liquid chromatography (RP-HPLC). The peptide sequences of YINQMPQKSRE, YIEAVNKVSPRAGQF, YINQMPQKSREA and VTGRFAGHPAAQ were identified by mass spectroscopy. While YIEAVNKVSPRAGQF represented the ACE-inhibitory activity, YINQMPQKSRE exhibited the highest antioxidant activity and DPP-4 inhibitory activity and VTGRFAGHPAAQ peptide showed anti-diabetic properties to α-glucosidase.

Antioxidant activity was investigated in a lipid oxidation system in some of the studies (Sakanaka and others 2004; Yousr and Howell 2015). Sakanaka and others (2004) studied the antioxidant effect of defatted egg yolk protein hydrolysates produced by enzymatic treatment. The hydrolysates showed the strongest antioxidant activity in lipid oxidation compared to the egg yolk protein and amino acid mixture of egg yolk. These hydrolysates were also tested in cookies with linoleic acid and they prevented the oxidation of polyunsaturated fatty acids. You and Wu (2011) aimed to produce egg protein (egg yolk and egg white) hydrolysates by enzymatic treatment with gastrointestinal and non-gastrointestinal enzymes (thermolysin and alcalase) and to fractionate the hydrolysates by ultrafiltration and cation exchange chromatography in order to study the antioxidant and ACE-inhibitory activities of peptides. The protein hydrolysates produced by thermolysin and alcalase showed a lower antioxidant activity compared to those produced by gastrointestinal enzymes. Antioxidant activity was correlated with low molecular weight peptides, especially those with a molecular weight of less than 500 kDa.

However, the above-mentioned chemical methods are not biologically relevant (Lopez-Alarcon and Denicola 2013; Chakrabarti and others 2014; Jahandideh and others 2015; Wang and others 2018). Cell-based studies have been considered a biologically relevant alternative to understand the antioxidant effect of proteins (Jahandideh and others 2015; Wang and others 2018). Wang and others (2018) aimed to purify and characterize the peptides from cooked egg and studied their antioxidant activity in A7r5 cells (vascular smooth muscle cell line), which is biologically relevant. The hydrolysates were produced using an *in vitro* model of the gastrointestinal digestion of humans. Afterwards, the peptides were fractionated and purified from the hydrolysates. The peptides showing the highest antioxidant properties were characterized by LC-MS/MS. DSTRTQ, DVYSF, and ESKPV produced from ovalbumin showed antioxidant activity in the cell line.

2.3.2. Anti-inflammatory activity of egg yolk hydrolysates

Inflammation is a response of the immune system to different stimulants such as pathogens, infection or tissue injury (Medzhitov 2008). Food bioactives with anti-inflammatory properties may have the potential to decrease the risk of chronic diseases such as cancer, type-2 diabetes and cardiovascular diseases. Egg yolk consumption in diet has different effects on inflammation such as metabolic syndrome or overweight in pro- or anti-inflammatory ways (Andersen 2015). The pro- and anti-inflammatory properties of egg yolk components such as phospholipids, cholesterol, the carotenoids lutein and zeaxanthin, and bioactive proteins were reviewed by Andersen (2015).

Studies based on peptides or hydrolysates derived from egg yolk proteins with anti-inflammatory activity are limited. Meram and Wu (2017) recently reported the effect of egg yolk livetins and their hydrolysates after enzymatic treatment on anti-inflammatory responses using *in vitro* models (lipopolysaccharide (LPS) induced RAW 264.7 macrophages). They controlled the reduction rate of inflammatory mediators such as nitric oxide (NO), prostaglandin-E2 (PGE2),

pro-inflammatory cytokines (tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6)) to understand the impact of livetins and hydrolysates on the prevention of inflammation (Meram and Wu 2017). In addition, the results from the reduction of inflammatory mediators were supported by the inhibition of the expression of some inflammatory enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Meram and Wu 2017). The findings demonstrated that livetins (α-, β-, and γ-livetin) and their hydrolysates obtained by hydrolysis with pepsin and alcalase resulted in a reduction of inflammatory responses, where especially the alcalase hydrolysates were more effective. Another study was conducted by Yoo and others (2017) to understand the effects of high hydrostatic pressure and enzymatic hydrolysis on the anti-inflammatory activity of egg yolk protein (phosvitin) hydrolysates in chemical and *in vitro* models (RAW 264.7 macrophages). Phosvitin hydrolysates obtained by alcalase and trypsin hydrolysis showed a decrease in NO production by macrophages. Inhibition of the production of pro-inflammatory cytokines was also observed by alcalase phosvitin hydrolysates. Phosphopeptides (PPPs) derived from egg yolk phosvitin showed an anti-inflammatory effect by reducing the expression of iNOS, TNF-α, IL-1β, and IL-6 in *in vitro* models (RAW 264.7 macrophages) (Xu and others 2012).

Only a few studies reported the effect of egg white proteins on anti-inflammatory responses. Lee and others (2017) studied the effect of vitelline membrane hydrolysates on anti-inflammatory activity in chemical and cellular models (RAW 264.7 macrophages), where the vitelline hydrolysates inhibited the production of NO and suppressed the upregulation of pro-inflammatory cytokines in the cellular model. Another study by Lee and others (2009) showed that egg white peptides decreased the local expression of some pro-inflammatory cytokines TNF-α, IL-6, IL-1β,

IFN-γ (interferon-gamma), IL-8 (interleukin-8), and IL-17 (interleukin-17) in porcine model of colitis.

CHAPTER 3. Effect of enzymatic hydrolysis on the extractability of cholesterol and phospholipids from egg yolk using supercritical CO_2

3.1. Introduction

The growing demand for high-quality egg white protein has led to a surplus of egg yolk, sold at much lower prices due to decreased demand. Egg yolk is an oil-in-water emulsion, containing 52% dry matter, in which lipids, proteins and others (carbohydrate, vitamins and minerals) account for 64%, 35% and 4%, respectively (Guilmineau and others 2005). It is important to fractionate the valuable bioactives in egg yolk and to characterize the fractions obtained for further value-added applications. Supercritical carbon dioxide (SC-CO_2) is a desirable solvent to extract lipids and bioactive compounds from different sources due to being non-flammable, non-toxic and leaving no solvent residue in the product.

Egg yolk lipids contain 62% triglycerides, 33% phospholipids (PL), and about 5% cholesterol. Cholesterol is a building block for hormones and vitamins and it is needed to build and maintain cell membranes. It is used in shrimp farming, and in nutraceutical and pharmaceutical products. On the other hand, phosphatidylcholine (PC) is a bioactive component in egg yolk, which accounts for 86% of total egg yolk phospholipids and this amount is three times more than that in soy phospholipids (Anton and others 2006). Choline has been added into infant formulas because it is an essential nutrient for liver function, brain development and cancer prevention (Gutierrez and others 1997). Hence, PC has broad applications in pharmaceutical and nutraceutical products, especially in infant formulas.

Several studies have reported the removal of cholesterol from dried egg yolk using SC-CO_2 (Froning and others 1990; Sun and others 1995; Warren and others 1991). Froning and others (1990) removed cholesterol from dried egg yolk using neat CO_2 at different operating conditions

(16.5 MPa/40 °C, 24.1 MPa/45 °C, 31.0 MPa/45 °C, 37.9 MPa/55 °C) without the removal of polar lipids. Increase in temperature and pressure simultaneously decreased the contents of lipids and cholesterol in egg yolk, which helped to produce lower fat and lower cholesterol egg yolk. Sun and others (1995) investigated the effect of the extraction temperature between 40 °C and 60 °C and pressure between 15 MPa and 35 MPa on the SC-CO_2 extraction of the total lipids and cholesterol from dried egg yolk. High temperature resulted in a high yield at 35 MPa, while the high pressures (25 and 35 MPa) also led to a high yield of egg yolk lipid extraction at constant temperature. Detailed review of the literature was provided in Chapter 2.

Several studies have been reported on the extraction of phospholipids from dried egg yolk using SC-CO_2 (Shah and others 2004; Aro and others 2009). Due to the polar nature of phospholipids, ethanol has been employed as a co-solvent. Aro and others (2009) developed a new method for the extraction of phospholipids with high-purity using ethanol as co-solvent at similar extraction conditions (45 MPa, 70 °C). For this purpose, they used an additional supercritical anti-solvent (SAS) process to precipitate the phospholipids and high purity PL (99%) was achieved. Navidghasemizad, Temelli and Wu (2014) reported an enzyme-assisted method of extraction where the egg yolk was enzymatically treated prior to extraction. The average recovery and purity of PC extracted from the cream after enzymatic hydrolysis were 94% and 57% at 48.3 MPa, 70 °C compared to 47% and 47% for dried egg yolk, respectively.

In the study of Navidghasemizad, Temelli and Wu (2014), the cholesterol content of the SC-CO_2 extracts was not determined. Determination of cholesterol amount in the extracts obtained over time should be performed to understand the extraction kinetics of cholesterol. In egg yolk, the majority of lipids is present as lipoproteins, mainly low density lipoproteins (LDLs). The assembly of LDLs was explained in Chapter 2. Enzymatic hydrolysis could be a viable approach

to degrade the structure of lipoproteins (Navidghasemizad, Temelli and Wu 2014). Lipases could hydrolyze the central lipid core while proteases could hydrolyze apoproteins. This could facilitate the release of the lipids (triglycerides, free fatty acids, phospholipids and cholesterol) and provide easy access to SC-CO_2 during the extraction process. Different combinations of proteolytic and lipolytic enzymes were tested to produce cream by Navidghasemizad, Acero-Lopez, Curtis, Temelli and Wu (2014). On the other hand, pellet preparation involves simply water dilution and centrifugation steps. Although pellet preparation is just a physical separation, it may have an effect on the lipoprotein structure.

Therefore, the aim was to take into consideration simultaneous extraction of high value compounds, especially phospholipids and cholesterol. The objective of this study was to determine the effect of pellet preparation and enzymatic hydrolysis on the extractability of neutral lipids (triglycerides, free fatty acids, cholesterol) and phospholipids from egg yolk using the two-step SC-CO_2 extraction process at 48.3 MPa, 70 °C, and CO_2 flow rate of 1.0 L/min (at ambient conditions) in the presence of 8% (mole%) ethanol as co-solvent.

3.2. Materials and methods

3.2.1. Pretreatment

3.2.1.1. Egg yolk pellet preparation

White shell eggs (grade A) were obtained from the Poultry Research Centre of the University of Alberta (Edmonton, AB, Canada). Egg yolks were separated from egg white manually by rolling on Whatman paper (grade No. 4) to remove albumen from the vitelline membrane, covering the egg yolk. The vitelline membrane was punctured with a sharp blade and egg yolk inside the membrane was collected into a beaker placed in an ice bath. Egg yolk pellet was prepared according to Kwan and others (1991) with some modifications. Egg yolk was diluted ten times

(w/w) with MilliQ water and pH was adjusted to 6 using 1 N HCl. The egg yolk slurry was mixed using a magnetic stirrer at 300 rpm for 1 h at 4 °C and then centrifuged at 10,000xg for 15 min at 4 °C to produce two different fractions (pellet and supernatant). The supernatant was separated from the pellet after centrifugation and the pellet was collected for enzymatic hydrolysis.

3.2.1.2. Enzymatic treatment

Egg yolk pellet was treated with several proteases and lipases in preliminary trials prior to the fractionation of neutral lipids and phospholipids using SC-CO_2. Cream formation was not achieved when Protease M and Lipase AY-30 were utilized individually. Their combination was tested by adding them sequentially with two-hour at each step; however, the cream formation was again not successful. Finally, a combination of Protease M and Lipase AY-30 was used simultaneously for further study due to the ease of the formation of a cream layer. A combination of Protease M (from *Aspergillus oryzae*, with an activity of 5,500 units/g, Amano Enzyme USA Co., Ltd., Elgin, IL, USA) and Lipase AY-30 (from *Candida rugosa,* a powder triacylglycerol lipase, active on short, medium and long chain fatty acids, Amano Enzyme USA Co., Ltd., Elgin, IL, USA) facilitated the formation of a cream layer. The aim was to simultaneously hydrolyze the triglycerides and cholesterol esters in the lipid core into free fatty acids, mono- and diglycerides, glycerol and cholesterol and to hydrolyze the apoproteins into peptides.

The fresh pellet after centrifugation was collected in a beaker and it was diluted six times (w/w) with MilliQ water according to Navidghasemizad, Temelli and Wu (2014). The pellet slurry was mixed using a magnetic stirrer at 300 rpm for 1 h at 4 °C and then hydrolyzed by a combination of Protease M and Lipase AY-30 at pH 5 and 50 °C for 3 h. Protease M and Lipase AY 30 were added simultaneously at the same level of 2% (w/w) based on the dry matter weight of egg yolk pellet. The dry matter of the pellet was analyzed gravimetrically over 16 h at 105 °C until the

sample weight became constant. For the hydrolysis of pellet, high-end titrator with 800 Dosino dosing system (Metrohm, model Titrando 842, Ionenstrasse, Switzerland) was used. The change in pH was recorded by using *tiamo*^{TM} database. The hydrolysis reaction mixture was held in a jacketed vessel to keep the temperature constant and the pH was held constant by dosing of 0.5 M HCl automatically while stirring at 300 rpm using a magnetic stirrer. After 3 h, hydrolysis was stopped by placing the vessel in an ice bath until the center temperature of slurry reached ambient temperature (23 °C). The hydrolysates were transferred to 50 mL plastic centrifuge tubes and centrifuged at 6,000xg for 30 min at 4 °C to separate cream (top layer) from the liquid subnatant (bottom layer). Separation was done by puncturing the bottom of the plastic centrifuge tubes using a sharp blade and removing the subnatant. The fresh cream was freeze dried (Labconco, model 7806020, Kansas, MO, USA), and stored at -20 °C until SC-CO_2 extraction. The pre-treatment steps of the whole process were summarized in Figure 3.1.

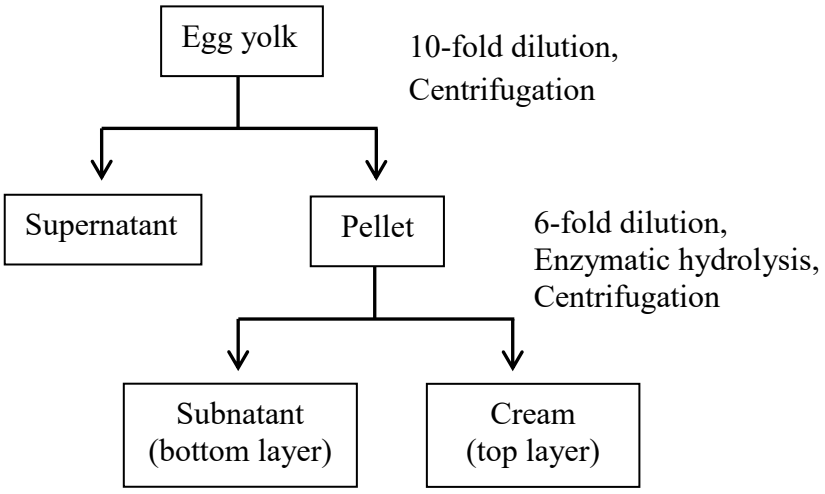

Figure 3.1. Flow chart of the pre-treatment of egg yolk, involving pellet preparation and enzymatic hydrolysis.

3.2.2. SC-CO_2 extraction process

A laboratory scale supercritical fluid extraction (SFE) unit (Newport Scientific Inc., Jessup, MD, USA) was used at Agri-Food Discovery Place. The flow diagram of the SFE unit was presented in Figure 3.2. Briefly, the basket was filled with the sample and placed into the extraction vessel. About 2 g of freeze-dried samples of yolk and pellet and 1.5 g of cream was mixed with about 10 g of glass beads (3 mm, Fischer Scientific, Pittsburgh, PA, USA) into the basket. The main reason to use glass beads was to enhance contact surface area and to limit channeling of CO_2. Yolk and pellet were placed into the basket with seven layers of sample and glass beads while cream was mixed with glass beads before placing them into the basket due to its sticky nature. About 1 g of glass wool was placed at both ends of the basket in order to prevent potential carry over of the sample with the flow of CO_2. CO_2 was pressurized to the target pressure and the temperature was kept constant with the heater around the extraction vessel, which was attached to a temperature controller. Upon depressurization of CO_2, extract fractions were collected in vials, which were kept in an ice-water jacket. After leaving the sample vials, CO_2 passed through the flow meter and a dry gas meter to measure the flow rate and volume of CO_2 used for the extraction process, respectively. The amount of fractions collected was determined gravimetrically.

The conditions were selected based on the previous study of Navidghasemizad, Temelli and Wu (2014) for the SC-CO_2 extraction process. Lipids were extracted in two steps at 48.3 MPa and 70 °C. CO_2 (Praxair Inc., Edmonton, AB, Canada, 99.9% (wt%) pure, bone and dry) flow rate was kept constant at 1 L/min, measured at ambient conditions. In the first step, neutral lipids, which are mainly triglycerides and cholesterol were extracted using neat CO_2 until the extraction curve reached a plateau. In this study, fractions were collected every 10 L of CO_2 consumption for the first 40 min. Then, the fractions were collected every 20 L of CO_2 consumption and the total run

time for the first step was 3 h. In the second step, polar lipids, mainly PL were extracted using CO_2 + ethanol. The ethanol was injected into the neat CO_2 flow using a high pressure piston pump (Model 305, Gilson Inc., Midleton, WI, USA). The level of ethanol addition was decided by following previous reports (Guclu-Ustundag and Temelli 2005; Navidghasemizad, Temelli and Wu 2014). Ethanol was added as a co-solvent at a flow rate of 0.2 mL/min in order to achieve a molar concentration of 8% ethanol in CO_2. The fractions were collected every 20 L of CO_2 consumption for 2 h. After that, the fractions were collected every 40 L of CO_2 consumption until the extraction curve reached a plateau. Total run time for the second step was about 6 h. The samples were dried under a gentle stream of nitrogen until constant weight was reached. The amount of extract fractions collected was determined gravimetrically.

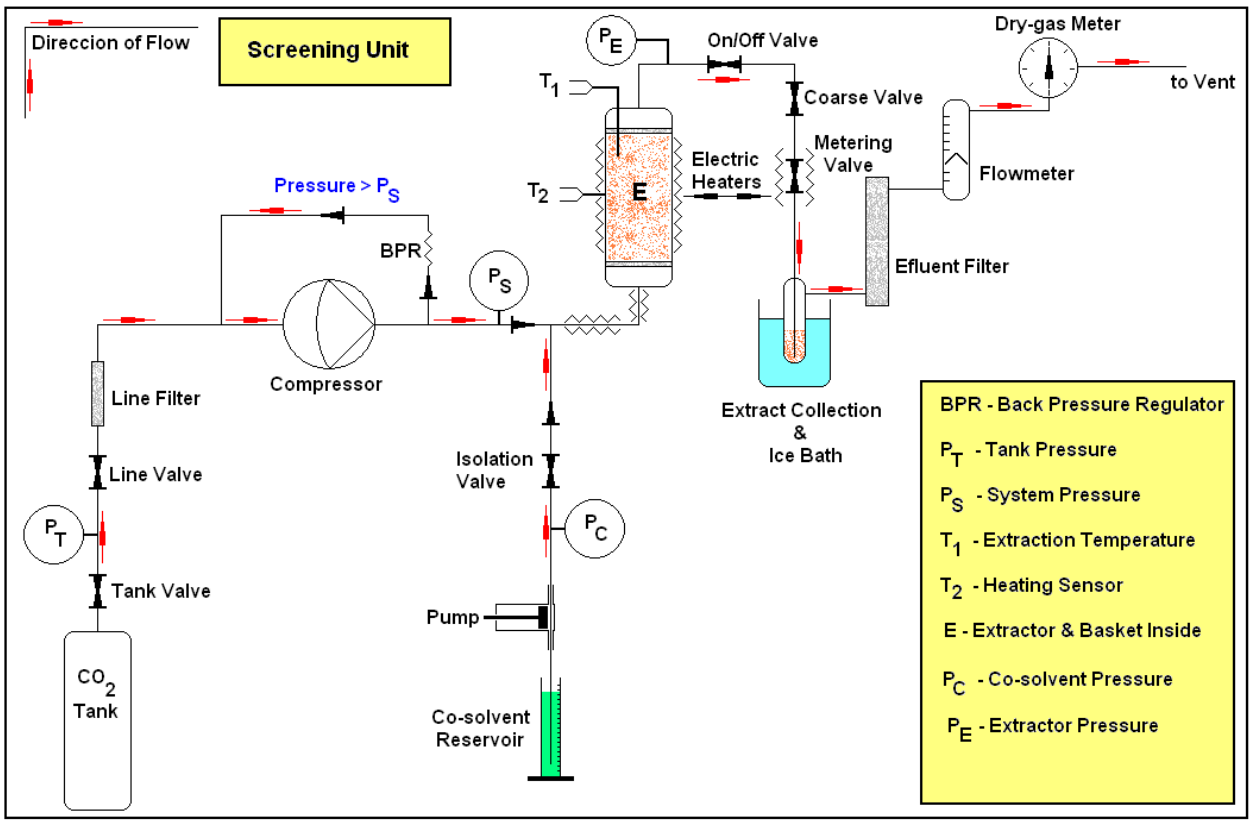

Figure 3.2. Flow diagram of the laboratory scale supercritical fluid extraction system.

3.2.3. Characterization of the fractions obtained

3.2.3.1. Proximate analyses

Cream, pellet and egg yolk were freeze dried. Moisture content was analyzed gravimetrically after 16 h at 105 °C until the sample weight became constant. Total lipid content was analyzed according to Hara and Radin (1978) with some modifications. About 1 g of dried sample was added to 18 mL hexane:isopropanol (3:2, v/v) and then the mixture was centrifuged at 6,000xg for 15 min. The upper layer was dried under a gentle stream of nitrogen and weighed gravimetrically. The total protein content was determined by Dumas combustion method using a TruSpec CN analyzer (Leco Corp., St Joseph, MI, USA). The protein content was calculated by multiplying the total nitrogen content with the conversion factor of 6.25. The results were reported as g/100 g on dry weight basis.

3.2.3.2. Cholesterol analysis and quantification

The cholesterol content of dried yolk, pellet and cream and their extract fractions collected over time during the two-step extraction (neat SC-CO_2 and SC-CO_2 + ethanol) were analyzed. Lipids were first extracted according to Section 3.2.3.1. Saponification step prior to the cholesterol analysis was done according to Fenton and Sim (1991) with some modifications. Lipids were mixed with 10 mL of KOH solution (9.4 mL 95% ethanol and 0.6 mL 33% KOH-water solution (w/v)) in glass tubes and capped tightly. Each tube was mixed for 1 min and heated at 60 °C in a water bath for 1 h with gentle shaking. Addition of 5 mL of MilliQ water and 10 mL of hexane to the glass tubes was done after the tubes were cooled to room temperature (23 °C). Each tube was mixed vigorously by a vortex mixer for 1 min. Upper hexane layer was transferred to glass vials, dried under a gentle nitrogen stream and stored at -20 °C until GC injection.

Samples were analyzed by Gas Chromatography-Mass Spectrometry (GC-MS). Identification of cholesterol was done by comparison with cholesterol standard (C8667 Sigma-Aldrich, Inc., St. Louis, MO, USA). The samples were prepared at a concentration of 1 mg/mL in hexane and analyzed directly. GC-MS analysis was performed using an Agilent 7890 GC (Agilent Technologies Inc., Santa Clara, CA, USA) system coupled with an Agilent 5975C MS (Agilent Technologies Inc., Santa Clara, CA, USA) mass spectrometer. Compounds were separated on a fused silica capillary column (Agilent HP-5, 30 m x 0.25 mm internal diameter, 0.25 μm film thickness; Agilent Technologies Inc., Santa Clara, CA, USA). Oven temperature was programmed at 200 °C for 1 min, and then increased to 300 °C with a ramp rate of 29 °C /min and held for 7.5 min. Run time was 13.5 min. Injector was kept at 250 °C. Split mode injection (1 μL) was done with a split ratio of 100:1. Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The scan range was 50-500 amu. The concentration of cholesterol in the samples was calculated using the calibration curve with a linear equation for the plot of peak area vs. concentration of cholesterol standard (C8667 Sigma-Aldrich, Inc., St. Louis, MO, USA).

3.2.3.3. Phospholipid analysis and quantification

Determination of PL classes (phosphatidylcholine (PC), phosphatidylethanolamine (PE) and lysophosphatidylcholine (LPC)) was established using high performance liquid chromatography (HPLC). Agilent 1100 HPLC instrument (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with Agilent 1100 quaternary pump (Agilent Technologies Inc., CA, USA) and Alltech ELSD 2000 Evaporative Light-Scattering Detector (Alltech Associates Inc., Lokeren, Belgium) was used according to Graeve and Janssen (2009) with some modifications. Briefly, lipids were extracted according to Section 3.2.3.1 from feed materials (yolk, pellet and cream) and the extract fractions collected over time during the second step of the SC-CO_2 extraction process for PL

analysis. The samples were dissolved in chloroform:isooctane:methanol (2:2:1, v/v/v) to solubilize the more polar phospholipids. Final total lipid concentration prior to injection was 10 mg/mL. About 1 to 10 μL samples were injected onto the column depending on the proportions of the various lipids present. A three-solvent gradient was used to separate the lipids on a 4.6 x 100 mm Onyx monolithic silica normal-phase column (Phenomenex Inc., Torrance, CA, USA). PL and cholesterol standards (egg yolk PC, egg yolk PE, synthetic 16:0 LPC, and sheep cholesterol) were used for quantification. The PL standards were purchased from Avanti Polar Lipids (Alabaster, AL, USA), and the cholesterol was from Sigma-Aldrich (St. Louis, MO, USA). PLs in the samples were identified by comparing the retention times of unknowns and standards. Individual calibration curves were produced for each lipid analyzed, and quantification was performed by using Agilent Chemstation software (Agilent Technologies Inc., Santa Clara, CA, USA).

3.2.3.4. Recovery calculation of cholesterol and PL in the extracts

Cholesterol and PL recoveries were calculated in two ways: based on the initial egg yolk required and the feed material used for SC-CO_2 extraction. The calculation was modified from Navidghasemizad, Temelli and Wu (2014). The first recovery was calculated based on the cholesterol or PL amount in the initial egg yolk considering the whole process using Eq. (1):

$$cholesterol\ or\ PL\ recovery\ (1) = \frac{\text{cholesterol or PL amount in extract (g) per } gram\ of\ feed\ used\ for\ extraction}{cholesterol\ or\ PL\ amount\ in\ egg\ yolk\ (g)\ to\ produce\ 1\ g\ of\ feed\ for\ extraction}\ x\ 100 \quad (1)$$

The second recovery was calculated based on the cholesterol or PL amount in the feed material used for SC-CO_2 extraction to better interpret the changes due to enzymatic hydrolysis using Eq. (2):

$$cholesterol\ or\ PL\ recovery\ (2) =$$

$$\frac{\text{cholesterol or PL amount in extract (g)} per\ gram\ of\ feed\ used\ for\ extraction}{\text{cholesterol or PL amount (g) in 1 } g\ of\ feed\ used\ for\ extraction} \ x\ 100 \qquad (2)$$

The purity of cholesterol in the extracts was calculated based on the amount of cholesterol in the extract fractions obtained during the first step of extraction using neat SC-CO_2 using Eq. (3):

$$cholesterol\ purity\ = \frac{\text{cholesterol amount in the extract (g)}}{\text{total amount of extract (g)}} \ x\ 100 \qquad (3)$$

3.2.3.5. Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE analysis of freeze-dried samples (yolk, pellet and cream) and the residue sample after SC-CO_2 extraction was run in 10-20% Mini-PROTEAN TGX precast protein gels using Mini-PROTEAN tetra cell unit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Freeze-dried samples (yolk, pellet and cream) were defatted with acetone for 1 h at 4 °C while residue samples (yolk, pellet and cream) after SC-CO_2 extraction were used directly. About 5 mg dried and defatted samples were dissolved in 750 µL MilliQ water and 250 µL of 20% SDS. Then, 60 µL of this protein solution was mixed with 60 µL Laemmli sample buffer (1:1, v:v) and the mixture was heated at 90 °C for 5 min. Each well of the gel was loaded with 15-30 µL of sample solution and electrophoresis was run at voltage of 150 V. Gels were stained in staining solution containing the mix of 30% methanol, 20% acetic acid and 0.1% Coomassie Brilliant Blue R-250 for 1 h and then gels were destained in destaining solution containing methanol, acetic acid and water (3:2:5, v/v/v). Finally, the protein bands were scanned by an Alpha Innotech gel scanner (Alpha Innotech Corp., San Leandro, CA, USA).

3.2.3.6. Morphology analysis by helium ion microscope

The morphology of egg yolk, pellet, and cream and their residues after SC-CO_2 extraction were analyzed using helium ion microscopy (HiM) (Zeiss Orion Helium Ion Microscope, Oberkochen,

BW, Germany) at the Nanofabrication and Characterization Facility (nanoFAB), University of Alberta. Freeze-dried samples of yolk, pellet and cream, and the residue samples after the two-step SC-CO_2 extraction process were used for HiM analyses. The residual ethanol was removed under a gentle stream of nitrogen at room temperature (23 °C).

3.2.3.7. Fatty acid composition

The fatty acid profiles of egg yolk, pellet, cream and their extracts obtained after the first step SC-CO_2 extraction and the second step SC-CO_2 + ethanol extraction were determined by GC-MS. Total lipids were extracted from egg yolk, pellet and cream prior to analysis according to Section 3.2.3.1. Fatty acids were methylated using 0.5 M methanolic sodium hydroxide for 2 h at 60 °C. After the addition of 2 mL of water and 3 mL of hexane, the tubes were centrifuged at 2,000xg for 5 min. The upper hexane layer was transferred to a 13x100 mm test tube containing a pinch of anhydrous sodium sulphate to remove residual water from the hexane layer by centrifugation at 2000 rpm for 2 min. About 1 mL of hexane layer was transferred to the screw cap GC vials to prevent evaporation. The fatty acids methyl esters (FAME) were stored at -20 °C until GC injection.

GC-MS was performed with an Agilent 7890A GC (Agilent Technologies Inc., Santa Clara, CA, USA) system coupled to an Agilent 5975C VL MSD (Agilent Technologies Inc., Santa Clara, CA, USA) mass spectrometer. Compounds were separated on a ZB-5MS fused silica capillary column (30 m x 0.25 mm internal diameter, 0.25 μm film thickness) (Phenomenex Inc., Torrance, CA, USA). The oven temperature program was 45 °C for 4 min, then 8 °C /min to 175 °C held for 1 min, 4 °C /min to 215 °C held for 2 min and 15 °C /min to 290 °C held for 6.75 min. The total run time was 45 min. Injector was kept at 250 °C. Split mode injection (1 μL) was used with a split ratio of 50:1. Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The spectrometers were operated in electron-impact (EI) mode. The scan range was 35-500 amu. GC-

MS data were analyzed using Agilent Technologies MassHunter Workstation Qualitative Analysis software (Agilent Technologies Inc., Santa Clara, CA, USA), where the peak areas were integrated. The library used for compound identification was NIST 08 GC-MS Database.

3.2.4. Statistical analyses

All experiments and analyses were performed in triplicates and the data were reported as mean ± standard deviation. One-way analysis of variance (ANOVA) using GraphPad Prism 6.02 (GraphPad Software, La Jolla, CA, USA) and Dunnet test for comparison of means against egg yolk were performed to determine the statistical differences between means at a confidence level of *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

3.3. Results and discussion

3.3.1. Proximate composition of dried egg yolk, pellet and cream

Total lipid, protein and cholesterol contents of dried yolk, pellet and cream samples were shown in Table 3.1. According to Kwan and others (1991), pellet after supernatant separation contains over 90% of the lipids in egg yolk, which is in agreement with the results of this study (Figure 3.3). The supernatant part contained water-soluble proteins (IgY, α- and β-livetins), while the pellet was rich in lipids. After enzymatic hydrolysis by Protease M and Lipase AY30 and following centrifugation, two different layers were produced with a top layer (cream), which was rich in lipids and a bottom layer (subnatant), which was rich in proteins. Hydrolyzing the pellet increased the lipid content of the cream while decreasing its protein content. Hydrolysis of the pellet by Protease M resulted in the hydrolysis of proteins into smaller peptides and they could be solubilized into the subnatant phase. The cream had less protein content (15%) compared to the yolk (32%) and the pellet (30%). On the other hand, lipids were concentrated in the cream, reaching a lipid content of 82%, compared to 63% in yolk and 66% in pellet. Cholesterol was also concentrated in the cream (4.8%, on dry matter basis) compared to yolk (3.5%) and pellet (3.4%).

Lipid content of pellet was higher (3% difference) while its protein content was lower (2% difference) compared to those of egg yolk.

Table 3.1. Protein and total lipid contents of feed materials used for SC-CO_2 extraction (g/100 g dry matter).[a] Cholesterol and individual PL were reported as wt% of total lipids.

Samples	Protein	Total lipid	Cholesterol	PC	PE	LPC
Yolk	32 ± 0.1	63 ± 1.0	5.5 ± 0.2	19.2 ± 2.0	1.8 ± 1.0	ND[b]
Pellet	30 ± 0.1	66 ± 0.5	5.2 ± 0.2	18.0 ± 2.4	3.9 ± 1.0	ND
Cream	15 ± 0.4	82 ± 0.9	5.1 ± 0.2	5.3 ± 2.5	1.5 ± 0.3	7.9 ± 0.4

[a] The results are expressed as mean ± standard deviation (n=3)
[b] Not detectable.

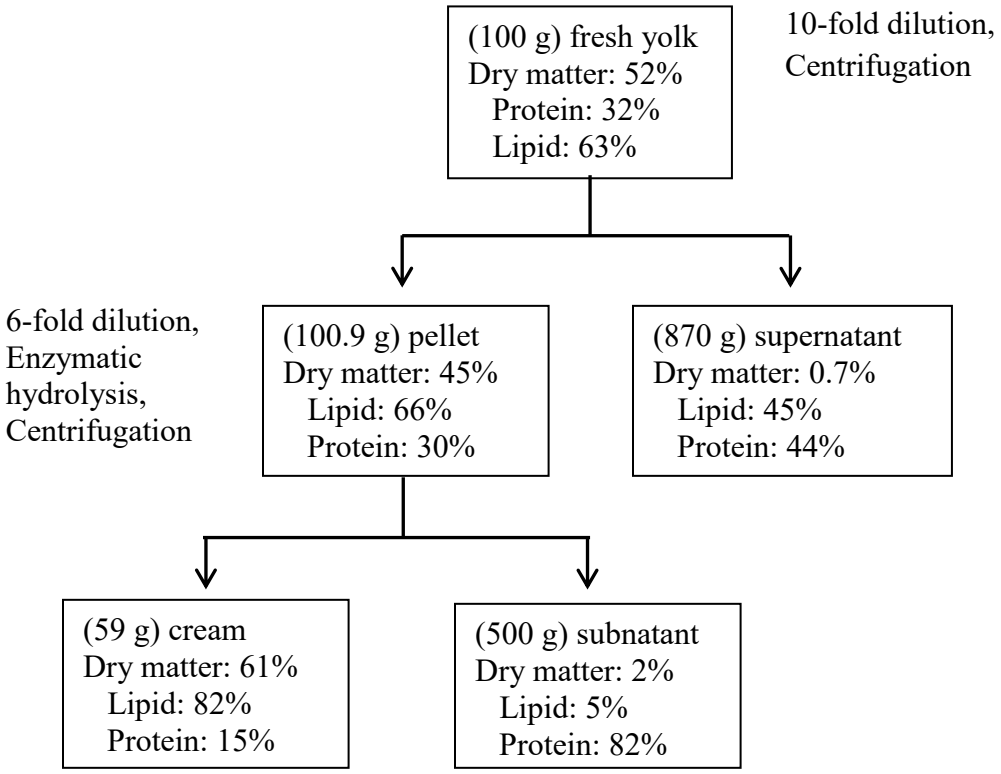

Figure 3.3. Mass balance of cream production from fresh egg yolk (% of lipid and protein were given on dry matter basis).

Egg yolk lipids consist of 62% triglycerides, 33% PL, and less than 5% cholesterol (Anton 2007a). About one-third of egg yolk lipids is PC (66-74%) (Rossi 2007). PC was also found to be the major PL in the cream and pellet (Table 3.1). While LPC was not detected in the yolk and pellet, 7.9% LPC was determined in the cream. Enzymatic treatment with lipase could have caused PC to be converted into LPC in the cream. Therefore, the PC content of the cream was lower than that of egg yolk. LPC makes up 3-6% of PL in egg yolk (Rossi 2007); however, LPC was not detected in egg yolk, which could be due to its low concentration that may be under the detection limit of the analysis protocol employed in this study.

3.3.2. SC-CO_2 extraction curves

Amounts of extracts for yolk, pellet and cream were presented cumulatively in Figure 3.4 using the two-step SC-CO_2 extraction process. A steep increase in the amount of extract was seen in the first 30 min of neat SC-CO_2 extraction while a gradual increase was observed for all extractions during the second step of the SC-CO_2 extraction process. In the first step, easily accessible lipids were solubilized and extracted by the neat CO_2 as reflected in the initial linear portion of the extraction curves for all treatments, which represents the solubility-controlled region. After the transition stage, the extraction curves reached a plateau, which indicated slowdown of extraction rate and transition to diffusion-controlled region when there were no more lipids left to be extracted using neat CO_2. In the second step, ethanol was introduced into SC-CO_2 as a co-solvent as indicated with the light grey symbols in Figure 3.4. Neutral lipids (triglycerides, free fatty acids and cholesterol) were extracted in the first step while polar lipids (phospholipids) were extracted in the second step in the presence of ethanol. The highest total extract yield was obtained from the cream (64%) while pellet (53%) showed a comparable yield with that of yolk (50%). The linear portion of the extraction curves was observed for the first 30 min (use of 2740 g of CO_2 per 100 g

feed) of extraction (Fig. 3.4). A large portion of lipids in the sample matrix was extracted in the initial linear region, corresponding to 90%, 80% and 83% of neutral lipids for the cream, yolk and pellet, respectively. For this calculation, the total extract amount obtained at the end of the linear portion of the extraction curve was divided by the total amount of lipids extracted at the end of the first step of extraction with neat CO_2.

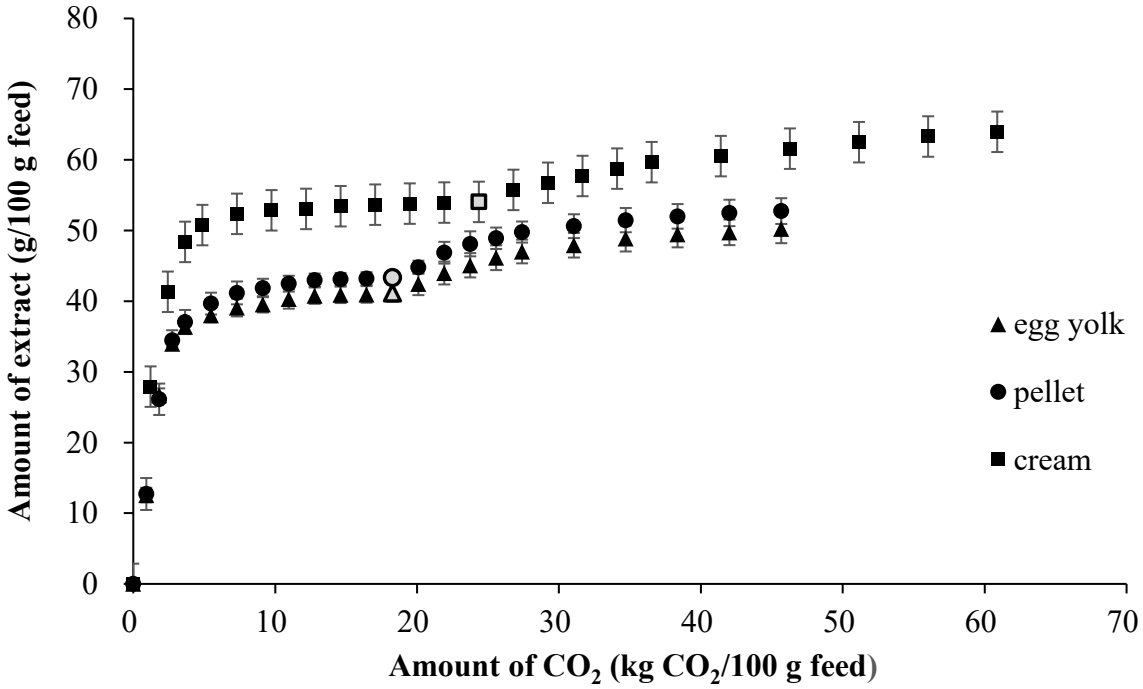

Figure 3.4. Total extract yield of the egg yolk, pellet and cream using neat SC-CO_2 in the first step and with addition of ethanol in the second step (Grey symbols represent the start of ethanol addition into SC-CO_2 as a co-solvent).

CO_2 loading (g extract/g CO_2) was calculated from the slope of the initial linear portion of the extraction curves (Fig. 3.5). The highest slope value (y=0.0242) was obtained for the cream, because easier access for the lipids in the cream provided shorter extraction time. During sample preparation, the cream was mixed with glass beads before filling the extraction basket while it was

filled with seven layers of glass beads and the sample for the egg yolk and pellet. The cream was a paste compared to the powder form of pellet and yolk. This situation also increased the contact surface area and CO_2 diffusion into the samples. This resulted in easy accessibility and extractability of lipids. The extraction curves for the pellet and the yolk (with slopes of y=0.0144 and y=0.0142, respectively) overlapped in this linear region, which showed that the CO_2 loadings of their lipids in SC-CO_2 were similar in the initial linear portion of the curves as shown in Figure 3.5.

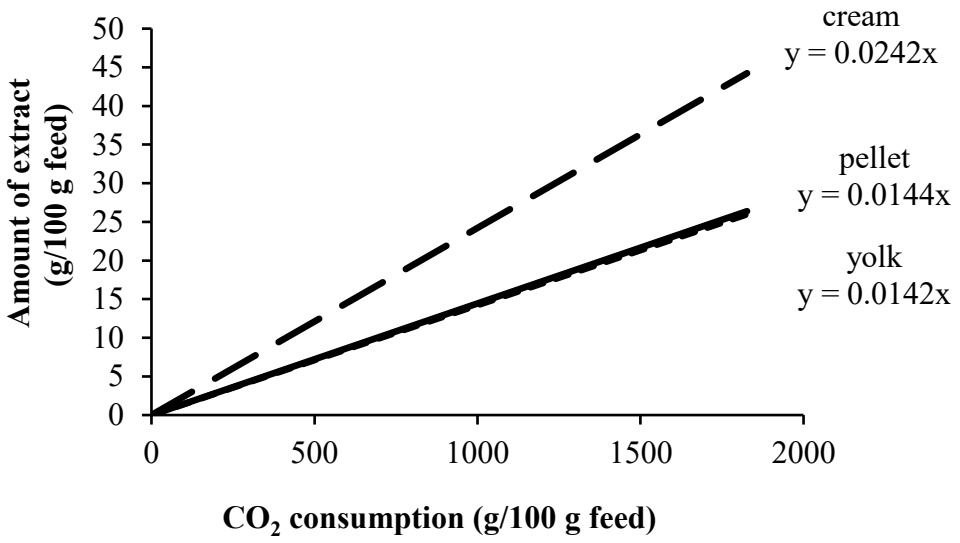

Figure 3.5. Slope of the initial linear portions of the extraction curves of the first step using neat SC-CO_2.

3.3.3. Cholesterol content of the extracts

No cholesterol was detected in the extracts obtained by SC-CO_2 + ethanol in the second step of the extraction; therefore, the following discussion focuses only on the first step of extraction. The average recovery of cholesterol for the whole process (Recovery 1) and the recovery based on the feed material used for the extraction (Recovery 2) were calculated using Eq. (1) and (2), and

the results were presented in Table 3.2 and Figure 3.6, respectively. The cholesterol concentrations of extracts obtained with neat SC-CO_2 were also calculated and presented in Table 3.2.

There was no significant difference ($p > 0.05$) between the cholesterol recoveries of yolk (63%) and pellet (62%) (Recovery 1). However, hydrolyzing the pellet by Protease M and lipase improved the cholesterol recovery significantly ($p < 0.01$) (83%). Warren and others (1991) reported that 98% of total cholesterol in dried egg yolk was removed at the highest temperature and pressure tested using neat SC-CO_2 (at 38 MPa and 55 °C). On the other hand, about two-thirds of the total cholesterol of dried egg yolk was removed at 31 MPa, 45 °C or 38 MPa, 55 °C using neat SC-CO_2 by Froning and others (1990) and 75% of dry yolk cholesterol was extracted regardless of the extraction conditions using SC-CO_2 by Paraskevopoulou, Panayiotou and Kiosseoglou (1997).

Considering Recovery 2 (Fig. 3.6), hydrolyzing the pellet with Protease M and lipase improved the extractability and the recovery of cholesterol (98%, 73% and 63% for cream, pellet and yolk, respectively). The recoveries of cholesterol from cream and pellet were significantly ($p < 0.01$ and $p < 0.05$, respectively) higher than that of yolk, indicating the importance of the structure of the feed material matrix on the extractability of cholesterol. Lipids are present as a complex with proteins mainly in LDL; structural changes in lipoproteins due to hydrolysis could facilitate diffusion of SC-CO_2 and release of lipids through the matrix. This was also explained by Navidghasemizad, Temelli and Wu (2014) for PL extraction from dry egg yolk using SC-CO_2. Furthermore, lipids were concentrated in the cream as the protein level was substantially decreased (Table 3.1). It should be noted that pellet preparation (water dilution and centrifugation) could also enhance its extractability due probably to the removal of water-soluble proteins interacting with lipoproteins, thus exposing lipoproteins for better extractability. This was reflected in the total

extract yield of pellet being higher than that for yolk. However, there was no significant difference ($p > 0.05$) between the cholesterol recoveries of yolk and pellet (Table 3.2). Furthermore, there was no significant difference ($p > 0.05$) between the purities of cholesterol in the extracts obtained from yolk, pellet and cream (Table 3.2). These results showed that more studies are needed on the separation of cholesterol from the total extract, which is a mixture of mono-, di- and triglycerides, free fatty acids, glycerol, cholesterol esters and cholesterol depending on the extent of enzymatic lipid hydrolysis.

Table 3.2. The cholesterol recovery and purity of the extracts using SC-CO_2 based on the amount of initial egg yolk considering the whole process (Recovery 1).[a]

Treatment	Cholesterol Recovery (1) (%)	Cholesterol Purity (%) (w/w)
Dry yolk	63 ± 4	6.0 ± 1.5
Dry pellet	62 ± 4	5.7 ± 0.9
Dry cream	83 ± 5**	7.4 ± 0.1

[a] The results are expressed as mean ± standard deviation (n=3)
** Significant difference between the treatments was determined by one way ANOVA with Dunnet's test at $p < 0.01$, indicating the difference compared to the yolk in the same column.

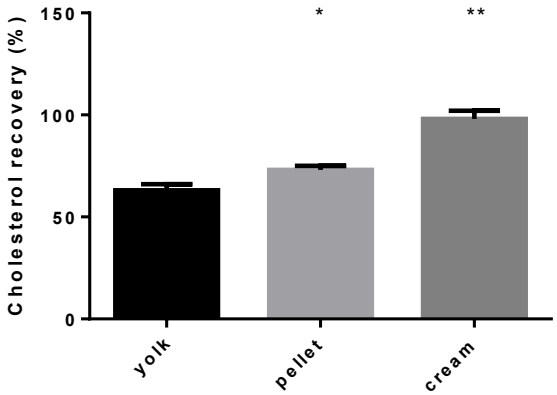

Figure 3.6. Cholesterol recovery of the extracts using SC-CO_2 based on the feed material used for extraction (Recovery 2) (*, ** indicate significant difference from that of the yolk at $p < 0.05$ and $p < 0.01$, respectively).

Determination of cholesterol in the different extract fractions is important to understand the extraction kinetics to potentially design further fractionation approaches. Previous studies described the collection of one extract at the end of the total extraction time and analyzed the cholesterol content of the total extract (Warren and others 1991; Froning and others 1990). In this study, extract fractions collected over time were analyzed separately, which showed that a large portion of total cholesterol was obtained at the beginning of the extraction period using neat SC-CO_2 during the first 30 min (Table 3.3).

Table 3.3. Distribution of cholesterol in the different fractions (cholesterol amount in each fraction/total amount of cholesterol extracted) collected based on time and amount of CO_2 used during the first step of neat SC-CO_2 extraction.

Type of extracts	Fraction number	Time (min)	Amount of CO_2 (g)	%[a]
Dry yolk	1	10	18.3	30 ± 7
	2	10	18.3	42 ± 0.1
	3	10	18.3	20 ± 7
	4	50	91.3	8.0 ± 1
	5	100	182.7	ND[b]
Dry pellet	1	10	18.3	30 ± 6
	2	10	18.3	34 ± 2
	3	10	18.3	22 ± 6
	4	50	91.3	14 ± 3
	5	100	182.7	ND
Dry cream	1	10	18.3	29 ± 5
	2	10	18.3	45 ± 4
	3	10	18.3	21 ± 4
	4	50	91.3	5.0 ± 3
	5	100	182.7	ND

[a] The results are expressed as mean ± standard deviation (n=3)
[b] ND= Not detectable.

After a certain period of time, no more cholesterol was extracted using neat SC-CO_2 and cholesterol in the feed material was exhausted. In general, all samples showed a similar pattern.

Second fractions of the extracts, which are collected between 10 and 20 min had the highest percentage of total cholesterol which was 42%, 34% and 45% for yolk, pellet and cream, respectively (Table 3.3). On the other hand, the final extract fractions did not contain any cholesterol. The total cholesterol extraction time was 80 min for all samples with the use of 146.2 g of CO_2.

3.3.4. Phospholipid content of the extracts

The recoveries of PL for the entire process and based on the feed material were calculated according to Eqs. (1) and (2), respectively. As expected, hydrolyzing the pellet by Protease M and Lipase AY30 improved the extractability of PL from the feed materials (Recovery 2) as shown in Figure 3.7. The recoveries were 93%, 40% and 32% for the cream, yolk and pellet, respectively. Navidghasemizad, Temelli and Wu (2014) also reported the recoveries of PC based on the feed material as 94% vs. 47% and 39% for dry cream hydrolyzed by Protease P and lipase, egg yolk and pellet, respectively. Unlike the cholesterol recovery results, there was no significant difference ($p > 0.05$) between the PL recoveries from the pellet and yolk based on the feed material. The PC recovery from pellet based on feed material was also reported by Navidghasemizad, Temelli and Wu (2014). The recovery based on the entire process (Recovery 1) was presented in Table 3.4. Cream showed the highest PL recovery (58%) compared to those for pellet (30%) and yolk (40%) for the entire process. Navidghasemizad, Temelli and Wu (2014) reported higher recoveries for PL, which was 72% for the cream after enzymatic treatment by the combination of Protease II and lipase and 85% for the combination of Protease P and lipase. Similarly, the pellet showed the lowest recovery (25%) for the entire process compared to the yolk in the previous study by Navidghasemizad, Temelli and Wu (2014), which is consistent with the results of this study. The cream had a lower total PL content compared to the egg yolk (Table 3.1), which resulted in a lower

recovery based on the entire process although the recovery of the cream was 93% based on the feed material used for extraction. On the other hand, Aro and others (2009) reported only 12-18% recovery of PC+PE from dry egg yolk using 12% ethanol as co-solvent at 70 °C, 40 MPa and 5 h extraction time with the two-step SC-CO_2 extraction.

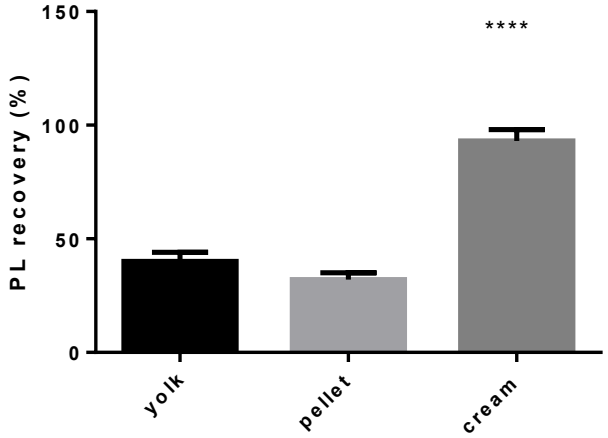

Figure 3.7. The PL recovery of the extracts using SC-CO_2 + ethanol based on the feed material used for extraction (Recovery 2) (**** indicates significant difference from that of the yolk at $p < 0.0001$).

Table 3.4. The PL recovery of the extracts obtained using SC-CO_2 + ethanol based on the amount of initial egg yolk considering the whole process (Recovery 1).[a]

Treatment	PL Recovery (1) (%)
Dry yolk	40 ± 4
Dry pellet	30 ± 4*
Dry cream	58 ± 3**

[a] The results are expressed as mean ± standard deviation (n=3)

*, ** Significant difference between the treatments and yolk as determined by one way ANOVA with Dunnet's test at $p < 0.05$ and $p < 0.01$, respectively.

3.3.5. Microstructure analysis

In order to examine the effect of the two-step SC-CO_2 extraction process and enzymatic treatment on the microstructure, the residue samples after SC-CO_2 extraction and feed materials (dry yolk, dry pellet and dry cream) were analyzed by helium ion microscopy (HiM). The effect of SC-CO_2 extraction and enzymatic treatment on the microstructure of egg yolk has not been reported previously, even though some results were reported on the microstructure of LDLs and granules. Granules consist of spherical structures with diameters between 0.3 and 2 μm (Anton 2013), which was in agreement with the results of this study. Granules mainly consist of LDL, HDL and phosvitin linked by phosphocalcic bridges (Anton 2013). HiM images displayed yolk as aggregated granules (Fig. 3.8a). Egg yolk also showed a dispersed hive-like structure with large clumps (Navidghasemizad and others 2015). Pellet showed tightly packed structures of the complex of LDLs, HDLs and phosvitin (Fig. 3.8b). Meram and others (2018) examined the effect of phosvitin extraction on the microstructure of yolk, granules and leftover granules and showed a packed structure for granules. LDLs are the main component of yolk dry matter, accounting for about 70% of egg yolk dry matter (Anton 2007a). After enzymatic hydrolysis, HiM results showed a degraded structure in Figure 3.8c, which could be explained by the effect of enzymes. The proteolytic enzyme could hydrolyze yolk proteins, especially apoproteins, into smaller peptides while lipase could hydrolyze the triglyceride core of LDLs (Navidghasemizad Temelli and Wu 2014). After SC-CO_2 extraction (Fig. 3.8d-f) dissociated individual particles (granules) were observed in the residue samples. The reason could be that SC-CO_2 basically removes the lipids, leaving behind a more concentrated protein structure. As lipid is removed, the remaining protein structures become more distinct. Less integrated structure was observed for the cream (Fig. 3.8f). Pellet and yolk showed more integration (Fig. 3.8d-e). The pellet residue had a more integrated granular structure (Fig. 3.8e) compared to that for the cream residue (Fig. 3.8f). This result could

be linked to the level of lipid still remaining in the yolk and pellet samples. This result was confirmed by the extraction yield, which was higher for the cream than that for the pellet and the yolk (Fig. 3.4). Lipids are responsible for the integration of protein structures.

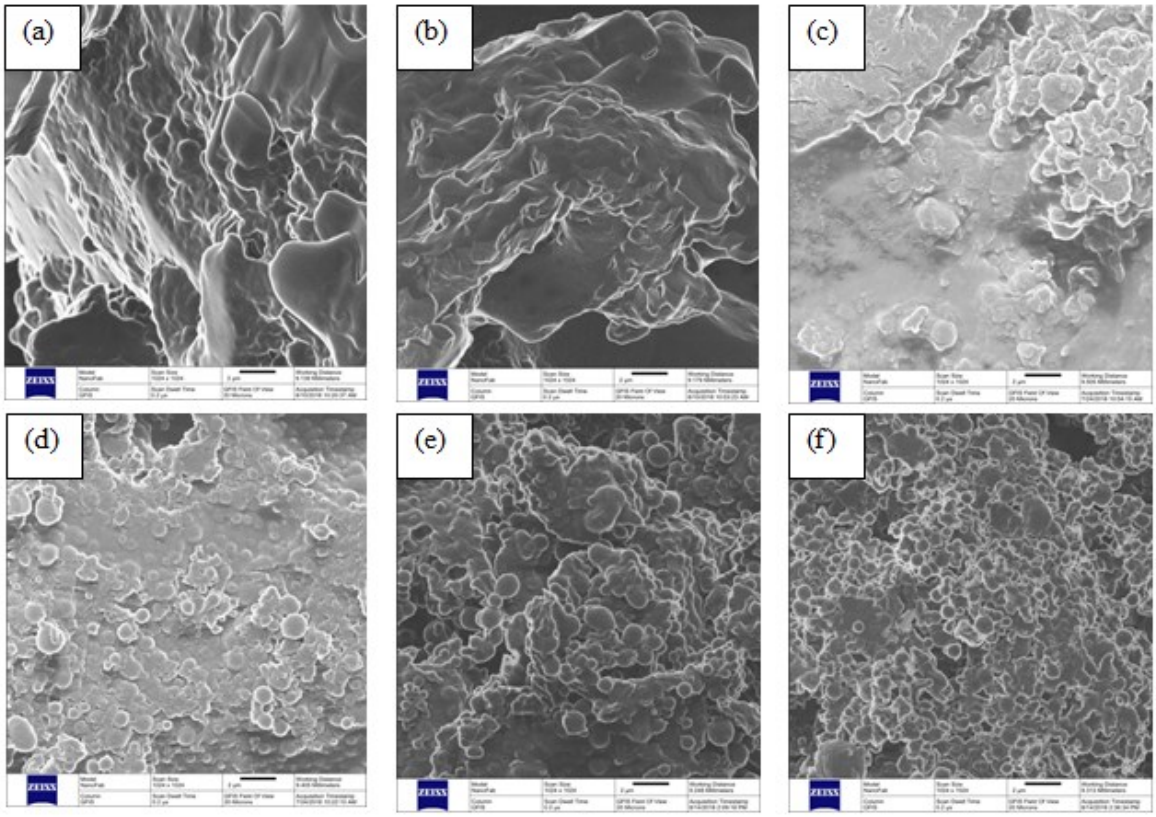

Figure 3.8. Helium ion microscopy (HiM) images of (a) Egg yolk (b) Pellet (c) Cream (d) Egg yolk after SC-CO_2 extraction (e) Pellet after SC-CO_2 extraction (f) Cream after SC-CO_2 extraction. Scale bars represent 2 μm for all images.

3.3.6. Fatty acid profile

The overall fatty acid profile was determined for the feed materials and their extracts obtained after the first step of extraction by neat SC-CO_2 and the second step of extraction by SC-CO_2 + ethanol as presented in Table 3.5. The main fatty acids found were oleic (C18:1), palmitic (C16:0), stearic (C18:0), linoleic (C18:2), palmitoleic (C16:1), arachidonic (AA) (C20:4, ω-6) and docosahexaenoic (DHA) (C22:6, ω-3) acids in yolk, pellet, cream and their extracts obtained by

SC-CO_2 extraction. Similar results were reported by Wang and others (2000). The dominant fatty acids reported generally were oleic (C18:1), palmitic (C16:0) and linoleic (C18:2) acids accounting for 40-45%, 20-25% and 15-20%, respectively (Kuksis 1992).

Overall, saturated fatty acids account for 30-35% while monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids make up 40-45% and 20-25%, respectively (Kuksis 1992). In this study, MUFA comprised 47% while saturated fatty acids and PUFA accounted for 42% and 11%, respectively, for egg yolk (Table 3.5). Fatty acid profiles of the three feed materials (yolk, pellet, cream) were similar, for example oleic acid (C18:1) content was 44%, 45% and 43% for yolk, pellet and cream, respectively. The MUFA content was higher while PUFA was lower in the extracts obtained with neat SC-CO_2 because AA and DHA were not detected in these extracts.

PC has an amphiphilic character, which has a non-polar part containing ω-3 fatty acids and a polar part containing choline. PC has long chain polyunsaturated fatty acids such as DHA and AA (Anton and others 2006). This was confirmed by comparing the fatty acid compositions of the extracts obtained with neat SC-CO_2 vs SC-CO_2 + ethanol. The results showed that AA (C20:4) and DHA (C22:6) were not detected in the neat SC-CO_2 extracts of yolk, pellet and cream. However, they were present in the extracts obtained in the second step using SC-CO_2 + ethanol at a higher ratio compared to their respective feed materials, accounting for 3% AA and 2% DHA in egg yolk and pellet extracts and 6% AA and 5% DHA in the cream extract. SC-CO_2 + ethanol extract of cream had a higher amount of AA and DHA compared to those from pellet and yolk because the PL recovery 2 based on the feed material for cream was the highest compared to those for yolk and pellet (Fig. 3.7). The highest PUFA amount was also obtained for the SC-CO_2 + ethanol extract of cream due to its higher AA and DHA contents.

Table 3.5. Overall fatty acid profiles[a] of feed materials used for extraction and their extracts after the two-step SC-CO_2 extraction.

	Fatty acids (%)									
Samples	**C16:1**	**C16:0**	**C18:2**	**C18:1**	**C18:0**	**C20:4**	**C22:6**	**SFA**	**MUFA**	**PUFA**
Yolk	2±0.6	29±1.2	10±0.5	44±1.4	13±1.4	1±0.2	1±0.1	42±3	47±2	11±1
Pellet	3±1	29±2	8±0.5	45±1	12±0.5	1±0.2	1±0.2	41±3	49±3	10±0.8
Cream	3±1	30±1	10±0.5	43±1	12±0.4	1±0.2	1±0.1	42±2	46±3	12±1
Yolk (neat SC-CO_2)	3±0.6	28±0.9	9±0.5	48±1.1	12±1.2	ND[c]	ND	40±2	51±2	9±0.5
Yolk (SC-CO_2 + ethanol)	1±0.0	33±0.4	10±0.2	35±0.4	16±0.6	3±0.2	2±0.1	49±1	36±0.4	15±0.5
Pellet (neat SC-CO_2)	4±0.3	27±0.9	8±0.5	50±1.3	10±1.1	ND	ND	37±1.9	55±1.5	8±0.5
Pellet (SC-CO_2 + ethanol)	1±0.0	36±0.6	9±0.3	31±0.6	17±0.7	3±0.2	2±0.2	53±1.2	32±0.6	15±0.7
Cream (neat SC-CO_2)	2±0.2	34±0.9	8±0.7	39±1.3	16±1.2	ND	ND	50±2.1	42±1.5	8±0.7
Cream (SC-CO_2 + ethanol)	2±0.0	21±0.1	12±0.1	43±0.3	13±0.1	6±0.0	5±0.0	33±0.3	44±0.3	22±0.1

[a] The results (GC peak area %) are expressed as mean ± standard deviation (n=3)
SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, and PUFA = polyunsaturated fatty acids.
ND= Not detectable.

3.3.7. SDS-PAGE profile

Figure 3.9 shows the SDS-PAGE profiles of egg yolk, pellet, cream and their residue samples after the two-step SC-CO_2 extraction process as well as the subnatant phase after enzymatic hydrolysis. The results clearly demonstrated that the enzymatic hydrolysis of the pellet resulted in smaller peptides in the cream compared to the pellet and the yolk. Major proteins in the yolk with molecular weights of 245, 120, 75, 69, 58, 48, 30, 13 kDa were hydrolyzed into small peptides. In general, the protein profiles of pellet and yolk were similar, indicating that the pellet preparation by water dilution and centrifugation did not change the protein profile of yolk. The protein profiles of the two-step SC-CO_2 extraction residues were similar to those of their respective feed materials (yolk, pellet and cream), demonstrating that SC-CO_2 extraction did not result in cleavage of

peptide bonds while removing lipids. Proteins are not soluble in SC-CO_2. However, the addition of ethanol in the second step could have an impact on the structure of some proteins. Changes in protein structure could be assessed by electrophoresis and liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS-MS) (Liao and others 2011); however, such information is not available for egg yolk proteins exposed to SC-CO_2 + ethanol treatment. On the other hand, as expected, protease treatment of pellet caused hydrolysis of proteins into small peptides, which ended up in the subnatant phase (Fig. 3.9, Lane 7). The largest protein fraction found in the subnatant phase was about 69 kDa and that for the residue cream sample after SC-CO_2 extraction was 110 kDa. The protein fractions observed in the subnatant phase were about 69, 48, 25 and 17 kDa. Cream residue sample also contained protein fractions with molecular weights of 69, 48, 30, 25, 20 and smaller than 11 kDa. Protein hydrolysis may generate small peptides having biological activities, which was the subject of the next study.

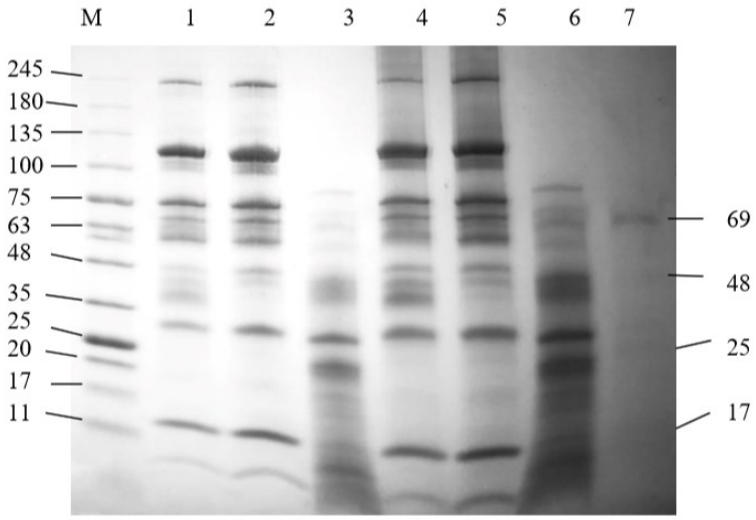

Figure 3.9. SDS-PAGE profile of feed materials (yolk, pellet, cream) and their residue samples after the two-step SC-CO_2 extraction process and subnatant after enzymatic treatment. Lane M: Standard molecular weight marker (11-245 kDa, Bio-Rad, Hercules, CA, USA); Lane 1: Yolk; Lane 2: Pellet; Lane 3: Cream; Lane 4: Yolk residue; Lane 5: Pellet residue; Lane 6: Cream residue; Lane 7: Subnatant phase after enzymatic hydrolysis.

3.4. Conclusions

A combination of Protease M and Lipase AY-30 treatment of egg yolk pellet facilitated the formation of a cream layer. The cream had a higher lipid content (82%) compared to egg yolk (63%). Lipids were extracted using SC-CO_2 in two steps at 48.3 MPa, 70 °C and CO_2 flow rate of 1.0 L/min (measured at ambient conditions); neutral lipids including cholesterol were extracted using neat CO_2 in the first step of extraction, while polar lipids were extracted in the second step with the addition of 8% (mole %) ethanol as co-solvent into SC-CO_2. Total extract yield (neutral lipids, cholesterol and phospholipids) from cream was higher than that from egg yolk. However, the increase in the purity of cholesterol in the extracts was not significant ($p > 0.05$) after enzymatic hydrolysis compared to that from dry yolk. Cholesterol was obtained within the neutral lipid fraction, which is a mixture of mono-, di- and triglycerides, free fatty acids, glycerol and cholesterol esters, depending on the extent of the enzymatic lipid hydrolysis. The purity of cholesterol in the neutral lipid fraction could be increased by adding another processing step at milder conditions where the free fatty acids can be removed from the mixture since they are more volatile and have a smaller molecular weight compared to the other lipid components present in the mixture. Hydrolyzing the pellet with Protease M and Lipase AY30 improved the extractability of cholesterol and PL. The recoveries of cholesterol and PL were 98% and 93%, respectively, based on the extraction feed material; however, it was 83% and 58% when the entire process was considered starting with egg yolk. PL were extracted using SC-CO_2 + ethanol and the fatty acid profile analysis of these extracts showed the presence of higher levels of DHA and AA. After SC-CO_2 extraction, the residue cream sample and the subnatant phase after hydrolysis were rich in small molecular weight peptides, which could have biological activities such as antioxidant

activity requiring further research. Fractionation of egg yolk creates potential new opportunities for value-added industrial applications.

CHAPTER 4. Antioxidant effect of egg yolk hydrolysates obtained after enzymatic treatment and supercritical CO_2 extraction in induced vascular smooth muscle cells (VSMCs) (A7r5) and the endothelial cell line (EA.hy926)

4.1. Introduction

Oxidative stress is an imbalance between the generation of reactive oxygen species (ROS) and the ability of the body to neutralize their effect. Studies show that oxidative stress is associated with obesity, cancer, cardiovascular diseases, inflammation, hypertension and ageing (Gutowski and Kowalczyk 2013). Inflammatory processes and oxidative stress cause progression of chronic diseases such as cancer and atherosclerosis (Chakrabarti and others 2014)**.** Chakrabarti and others (2014) reviewed bioactive peptides from food sources for beneficial effects such as antioxidant properties. Egg protein ovotransferrin (egg white protein) derived tripeptides (IRW and IQW) were found to be effective in exhibition of antioxidant effect by reduction of tumor necrosis factor (TNF) induced superoxide generation in endothelial cells (Majumder and others 2013). Another study also showed that IRW tripeptide inhibited TNF-α induced inflammatory response and oxidative stress in endothelial cells (Huang and others 2010). Nimalaratne and Wu (2015) reviewed the antioxidant compounds in egg yolk, including phosvitin, phospholipids, carotenoids, vitamin E and aromatic amino acids.

Chemical methods have been commonly used to assess the antioxidant activity such as oxygen radical absorbance capacity-fluorescein (ORAC-FL) assay, ABT (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate)) radical anion scavenging activity, DPPH (1,1-diphenyl-2-picryl-hydrazyl) radical scavenging activity and reducing power (RP) (Nimalaratne and others 2015; Liu and others 2015; Huang and others 2012; Wang and others 2018). However, these chemical methods were criticized due to the lack of biological relevance (Jahandideh and others

2015). Antioxidant activity was commonly measured using *in vitro* chemical-based assays; however, these methods do not consider the complexity of the *in vivo* systems (Jahandideh and others 2015; Wang and others 2018). Cell-based assays could be useful in assessing the potential antioxidant activity of a sample prior to *in vivo* studies due to being less costly and less time consuming (Lopez-Alarcon and Denicola 2013).

Egg yolk derived antioxidant peptides have been reported (Duan and others 2014; Yousr and Howell 2015; Yoo and others 2017). Egg yolk phosvitin was hydrolyzed by enzymes at different pressures and antioxidant activity was measured in chemical- and cellular-based models (Yoo and others 2017). The antioxidant activity of peptides from egg yolk protein was studied in chemical-based models by Yousr and Howell (2015). The peptides were isolated and purified from egg yolk protein and some fractions showed antioxidant activity. Zambrowicz and others (2015) investigated the antioxidant activity of egg yolk protein hydrolysates by chemical methods. The proteolytic digests of phosvitin from egg yolk was also studied for antioxidant activity (Duan and others 2014) using chemical-based methods.

There is an interrelation between oxidative stress and inflammatory responses (Jahandideh and others 2015). Vascular cells were mostly used for the determination of antioxidant and anti-inflammatory activities of egg white protein ovotransferrin-derived tripeptides (Huang and others 2010; Majumder and others 2013; Liao and others 2016). The anti-inflammatory properties were also found in connection with antioxidant activity using chemical methods (Huang and others 2012). Meram and Wu (2017) studied the effect of egg yolk livetins and their hydrolysates, prepared by proteolytic hydrolysis, on the inflammatory responses in lipopolysaccharide (LPS) stimulated murine RAW 267.7 macrophage *in vitro* model.

There is a lack of studies that investigate the potential antioxidant properties of hydrolysates obtained from egg yolk proteins using cell-based techniques. Egg yolk cream was produced after hydrolyzing the pellet using a combination of protease and lipase as reported in Chapter 3. Subnatant was produced as a residue after the enzymatic treatment of the pellet with a high content of low molecular weight proteins/peptides. Lipids (neutral lipids, phospholipids and cholesterol) were extracted from the egg yolk cream by a two-step extraction process using neat supercritical carbon dioxide (SC-CO_2) and SC-CO_2 + ethanol. The extraction residue was also rich in proteins with a low molecular weight (Chapter 3). There is a need to investigate the effect of these hydrolysates obtained from egg yolk proteins on oxidative stress since information on their potential antioxidant properties using cellular assays is not available. Therefore, the objective of this study was to determine the antioxidant activity of egg yolk hydrolysates obtained by enzymatic treatment and the two-step SC-CO_2 extraction process in induced vascular muscle cells (A7r5) and endothelial cell line (EA.hy926).

4.2. Materials and methods

4.2.1. Pretreatment

4.2.1.1. Egg yolk pellet preparation and enzymatic treatment

Egg yolk pellet was prepared according to Kwan and others (1991) with some modifications as explained in Section 3.2.1.1. Egg yolk pellet was treated with the combination of Protease M and Lipase AY-30 as explained in Section 3.2.1.2 to form cream and subnatant prior to the extraction of lipids from the cream using SC-CO_2. The subnatant phase was freeze dried and stored at -20 °C prior to the treatment of the cells.

4.2.2.2. SC-CO_2 extraction process

A laboratory scale supercritical fluid extraction unit (Newport Scientific Inc., Jessup, MD, USA) located at Agri-Food Discovery Place was used to extract lipids from the cream as explained in Section 3.2.2. Lipids were extracted in two steps at 48.3 MPa, 70 °C in the presence of ethanol as co-solvent in the second step. The residue sample of cream after SC-CO_2 extraction was dried under a gentle stream of nitrogen to remove residual ethanol from the sample until constant weight was reached. The cream residue was used for the treatment of the cells.

4.2.2. Cell culture

Rat aortic vascular smooth muscle cells (VSMCs) A7r5 (CRL-1444) and the human endothelial cell line EA.hy926 (CRL-2922) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM) (Gibco/Invitrogen, Carlsbad, CA, USA) was used to culture the cells. The cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco/Invitrogen, Carlsbad, CA, USA) and 1% antibiotics (penicillin, streptomycin and gentamicin) (Life Technologies, Carlsbad, CA, USA) until they reached 80% confluence. Cells were incubated at 37 °C in a humidified atmosphere (5% CO_2, 80% humidity). For experiments, the confluent cells were placed in a quiescing medium (DMEM + 1% FBS + 1% antibiotics) and then treated with 250, 500, 1000 μg/mL of the cream residue and the subnatant 1 h prior to the addition of 1 μM of Angiotensin II (Ang II) (Sigma-Aldrich, St. Louis, MO, USA) and recombinant human TNF-α (R&D system, Minneapolis, MN, USA) for different time periods. Cells between passages 4 and 11 were used for all experiments.

4.2.3. Cell viability assay

The number of viable cells present was performed according to the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) based calorimetric assay. Before the MTT assay, the number of viable cells was quantified by an automated cell counter (TC20, Bio-Rad

Laboratories, Inc., Hercules, CA, USA). The cells were pipetted into a 96-well flat bottom plate incubated at 37 ºC in a 5% CO_2 incubator until they reached 80% confluence. Then, DMEM media was removed, and the cells were treated with different concentrations (250, 500, 1000 µg/mL) of the cream residue and the subnatant for 24 h. MTT solution was prepared with thiazolyl blue tetrazolium bromide (M5655, Sigma-Aldrich, Inc., St. Louis, MO, USA) at the concentration of 5 mg/mL. At the end of the incubation time, 10 µL MTT solution was added to each well and then incubated for an additional 3 h. After that, the media was discarded and 100 µL dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Inc., St. Louis, MO, USA) was added to each well, and the plate was shaken for 10 min. The optical density of each well was determined by a SpectraMax M3 multimode microplate reader (Molecular Devices, Inc., Sunnyvale, CA, USA) at 570 nm. The percentage of viable cells was calculated compared to the control wells, containing only DMEM.

4.2.4. Determination of antioxidant activity (Superoxide Detection)

Cellular superoxide generation was detected by dihydroethidium (DHE) staining (10057, Biotium, Inc., Fremont, CA, USA) (Huang and others 2010). In principle, reactive oxygen species (ROS) react with DHE to generate ethidium, which binds to nuclear DNA and release fluorescence (Peshavariya and others 2007). The cells were seeded into a 48-well plate and grown in DMEM with 10% of FBS until confluent. After that, 250, 500 and 1000 µg/mL samples (the cream residue and the subnatant) were prepared in quiescing medium. The samples were added to the wells and incubated at 37 °C in a 5% CO_2 incubator for 1 h. The cotreatment period of the samples and Ang II (for A7r5) and TNF-α (for EA.hy926) was 30 min. Then, the cells were treated with 20 µM of DHE and incubated in the dark for 30 min, followed by washing three times with quiescing medium. The fluorescence signal was detected by Olympus IX81 fluorescent microscope (Olympus Corporation, Shinjuku, Tokyo, Japan). Images were taken from 3 random fields. Mean

fluorescence intensity (MFI) of each image was quantified by ImageJ software (http://imagej.net/Welcome). MFI/cell was calculated based on the cell number in each field. Results were reported as % of the untreated group.

4.2.5. Statistical analyses

The data were reported as mean ± SD (standard deviation) of 4 to 6 independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) using GraphPad Prism 6.02 (GraphPad Software, La Jolla, CA, USA) and Dunnet test for comparison of means. The statistical differences between means were given at a confidence level of *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

4.3. Results

The subnatant phase was produced prior to the extraction of lipids from the cream using SC-CO_2. The cream residue and the subnatant phase contained small protein fractions, as reported in Chapter 3 based on SDS-PAGE analysis (Fig. 3.9). The small protein fractions observed in the subnatant phase were about 69, 48, 25 and 17 kDa. Cream residue sample also contained small protein fractions with molecular weights of 69, 48, 30, 25, 20 and smaller than 11 kDa. The largest protein fraction found in the cream residue after SC-CO_2 extraction was 110 kDa while that for the subnatant was 69 kDa.

4.3.1. Determination of antioxidant activity in Ang II induced VSMCs (A7r5)

4.3.1.1. Effect of the subnatant and cream residue on the viability of VSMCs (A7r5)

MTT assay was used to determine the effect of the subnatant and cream residue on cell viability. As shown in Figure 4.1, the application of different concentrations (250 to 1000 μg/mL) of samples in the cell viability experiments did not exhibit any cytotoxic effects on VSMCs (A7r5)

compared to the control (cells without samples). Therefore, the antioxidant assays were carried out at these concentrations (250 to 1000 µg/mL).

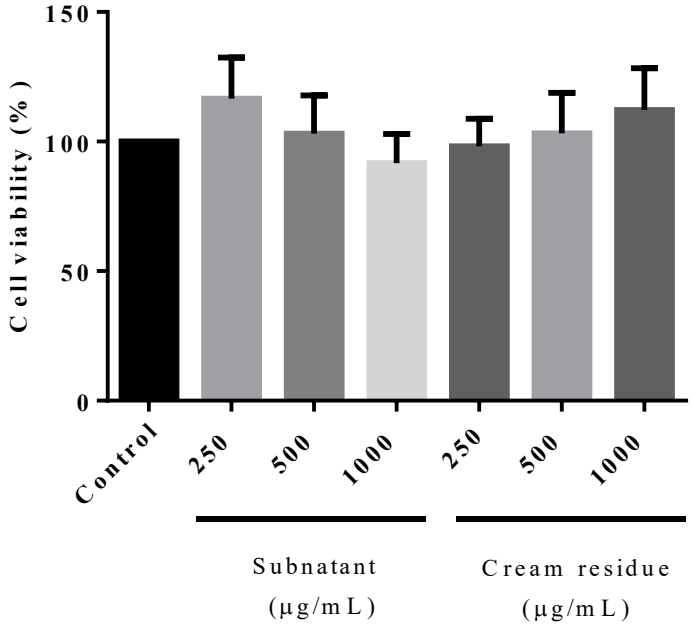

Figure 4.1. Effect of subnatant phase and cream residue on viability of VSMCs. A7r5 cells were treated with various concentrations of sample for 24 h. Cell viability was measured by MTT assay. Data are presented as mean ± standard deviation (n=6). There is no statistical difference ($p > 0.05$) between the control and treated groups as evaluated by one way ANOVA with Dunnett's test.

4.3.1.2. Effect of the cream residue and the subnatant on oxidative stress in Ang II induced VSMCs (A7r5)

The effect of the cream residue and subnatant on the intracellular superoxide generation was examined with or without Ang II stimulation. The fluorescence (ROS-mediated) was significantly increased by Ang II stimulation, which could then be suppressed by the sample treatments (Fig. 4.2). The effect of different concentrations of the subnatant on the superoxide level in VSMCs was shown in Figure 4.2. The addition of subnatant at concentration levels of 1000 and 500 µg/mL showed an antioxidant effect in VSMCs against Ang II by decreasing the ROS-mediated

fluorescence. However, at the lowest concentration tested (250 µg/mL), the subnatant did not have an effect ($p > 0.05$) on the superoxide level (Fig. 4.2). On the other hand, the effects of the two samples, cream residue and subnatant on Ang II stimulated oxidative stress in VSMCs were compared in Figure 4.3 at the highest concentration of 1000 µg/mL. The cream residue did not have an effect ($p > 0.05$) on the superoxide production while the subnatant significantly ($p < 0.01$) decreased the superoxide production. In conclusion, the cream residue did not show an antioxidant effect while the subnatant had antioxidant activity at levels of 500 and 1000 µg/mL.

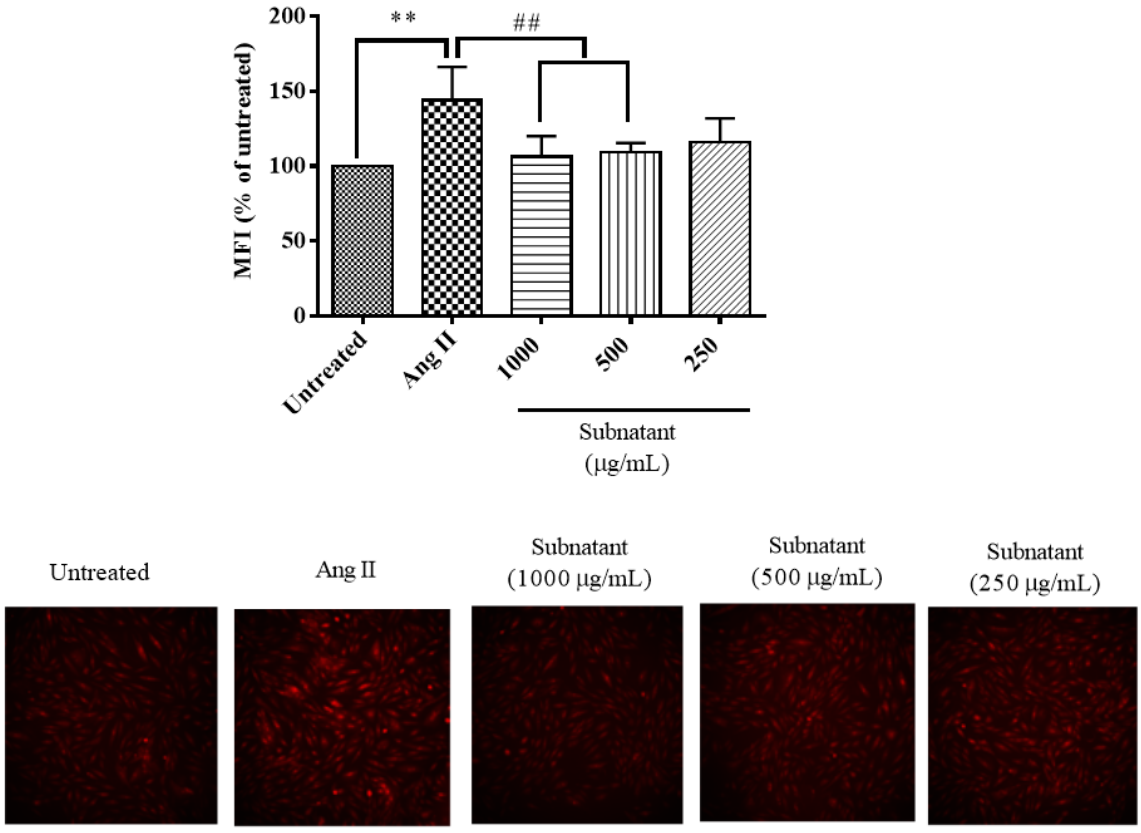

Figure 4.2. The subnatant at concentrations of 500 and 1000 µg/mL abolished Ang II stimulated oxidative stress in VSMCs. A7r5 cells were treated with 50 µM of different concentrations of the subnatant. Cells were treated with 10 µM of DHE for 30 min and the fluorescence signal was detected by fluorescence microscopy. Mean fluorescence intensity (MFI) was calculated based on the cell number and the data for each image. The data were shown as % of the untreated group. Data are presented as mean ± standard deviation based on 4 to 5 independent experiments. ** indicates $p < 0.01$ as compared to the untreated group, ## indicates $p < 0.01$ as compared to the Ang II treated group.

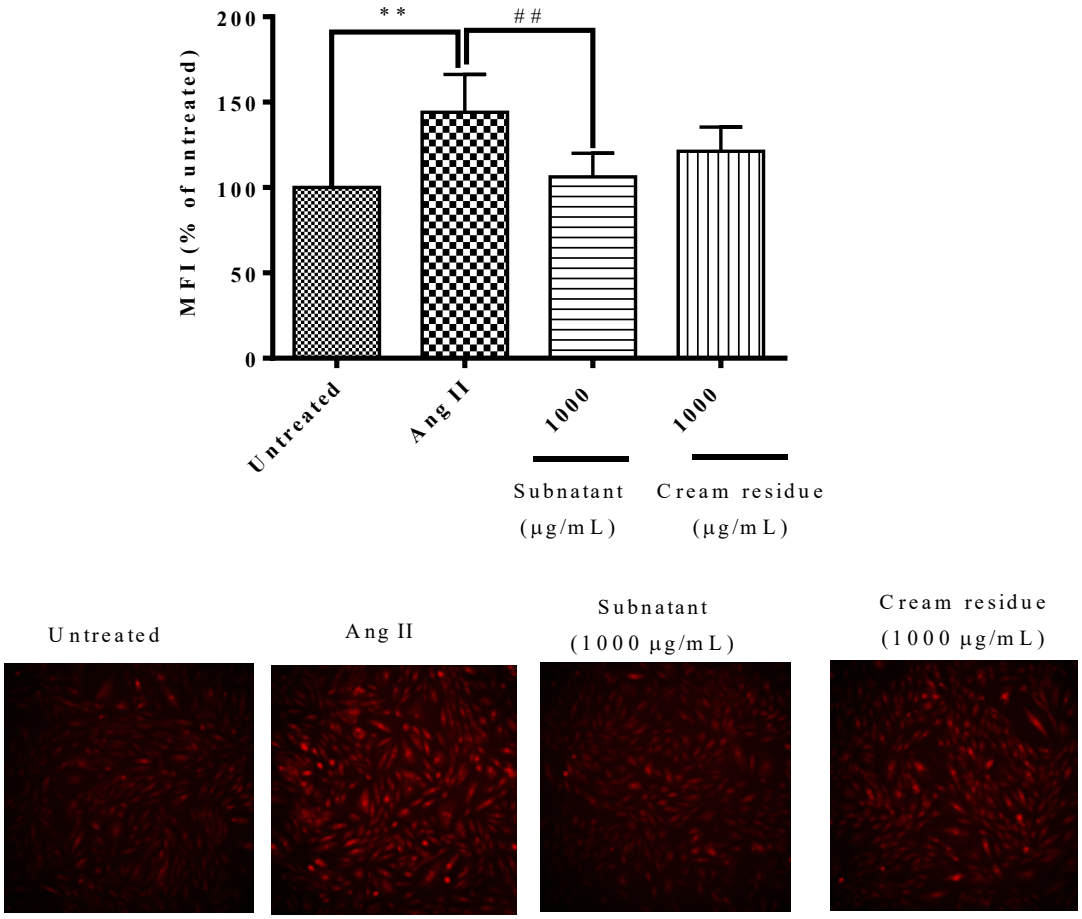

Figure 4.3. Effect of the subnatant and the cream residue at the fixed concentration of 1000 µg/mL on Ang II stimulated oxidative stress in VSMCs. A7r5 cells were treated with 50 µM of different concentrations of the subnatant. Cells were treated with 10 µM of DHE for 30 min and the fluorescence signal was detected by fluorescence microscopy. Mean fluorescence intensity (MFI) was calculated based on the cell number and the data for each image. The data were shown as % of the untreated group. Data are presented as mean ± standard deviation based on 4 to 5 independent experiments. ** indicates $p < 0.01$ as compared to the untreated group, ## indicates $p < 0.01$ as compared to the Ang II treated group.

4.3.2. Determination of antioxidant activity in the endothelial cell line EA.hy926

4.3.2.1. Effect of the subnatant and cream residue on the viability of the endothelial cell line EA.hy926

As shown in Figure 4.4, the application of different concentrations (250 to 1000 µg/mL) of the subnatant and cream residue samples in the cell viability experiments did not exhibit any cytotoxic

effects on the endothelial cell line (EA.hy926) compared to the control (cells without samples). Therefore, antioxidant assays were carried out at these concentrations (250 to 1000 µg/mL).

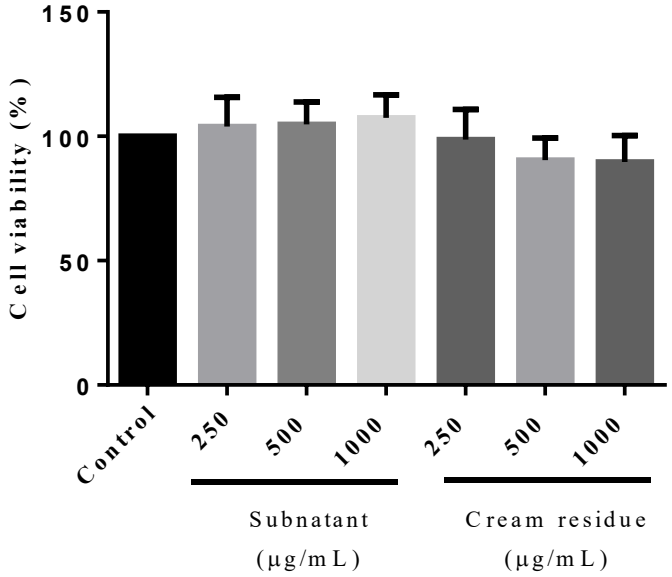

Figure 4.4. Effect of subnatant phase and cream residue on the viability of the endothelial cell line (EA.hy926). EA.hy926 cells were treated with various concentrations of sample for 24 h. Cell viability was measured by MTT assay. Data are presented as mean ± standard deviation (n=6). No statistical difference ($p > 0.05$) was found between the control and treated groups as evaluated by one way ANOVA with Dunnett's test.

4.3.2.2. Effect of the cream residue and subnatant on oxidative stress in TNF-α induced endothelial cells (EA.hy926)

The effects of the cream residue and the subnatant on the intracellular superoxide generation were examined with or without TNF stimulation in endothelial cells (EA.hy926). TNF-α stimulation significantly ($p < 0.01$) enhanced the ROS-mediated fluorescence while its effect was abolished by the subnatant treatment at some levels. The subnatant at the concentration level of 1000 µg/mL showed an antioxidant effect in endothelial cells against TNF-α by significant ($p < 0.05$) suppression of the ROS-mediated fluorescence (Fig. 4.5). The cream residue (1000 µg/mL) did not have an effect ($p > 0.05$) on the superoxide level in endothelial cells against TNF-α

stimulation (Fig. 4.6). The subnatant was effective at certain levels on the suppression of superoxide generation for both cells. The reason could be that the subnatant has small protein fractions.

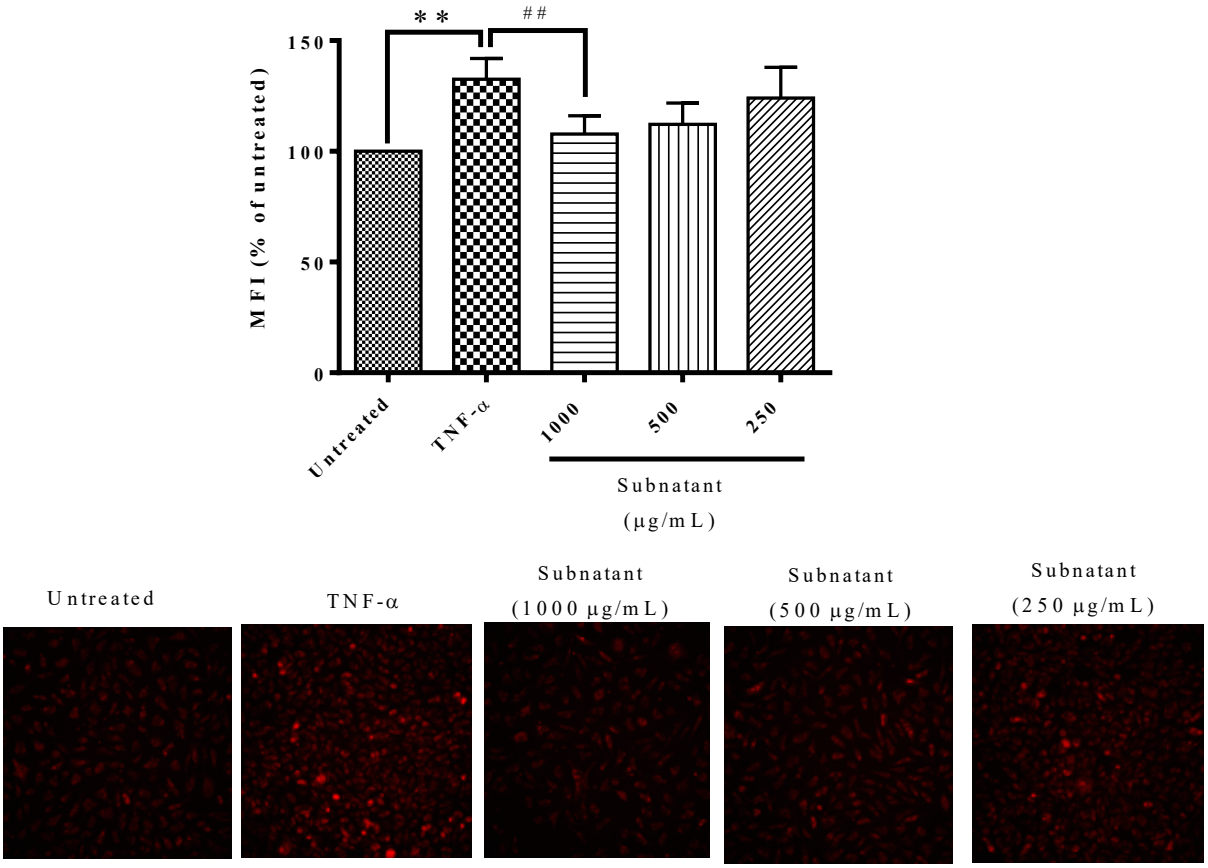

Figure 4.5. The subnatant at a concentration of 1000 μg/mL abolished TNF-α stimulated oxidative stress in endothelial cells. EA.hy926 cells were treated with 50 μM of different concentrations of the subnatant. Cells were treated with 10 μM of DHE for 30 min and the fluorescence signal was detected by fluorescence microscopy. Mean fluorescence intensity (MFI) was calculated based on the cell number and the data for each image. The data were shown as % of the untreated group Data are presented as mean ± standard deviation based on 4 independent experiments. ** indicates $p < 0.01$ as compared to the untreated group, ## indicates $p < 0.01$ as compared to the TNF-α treated group.

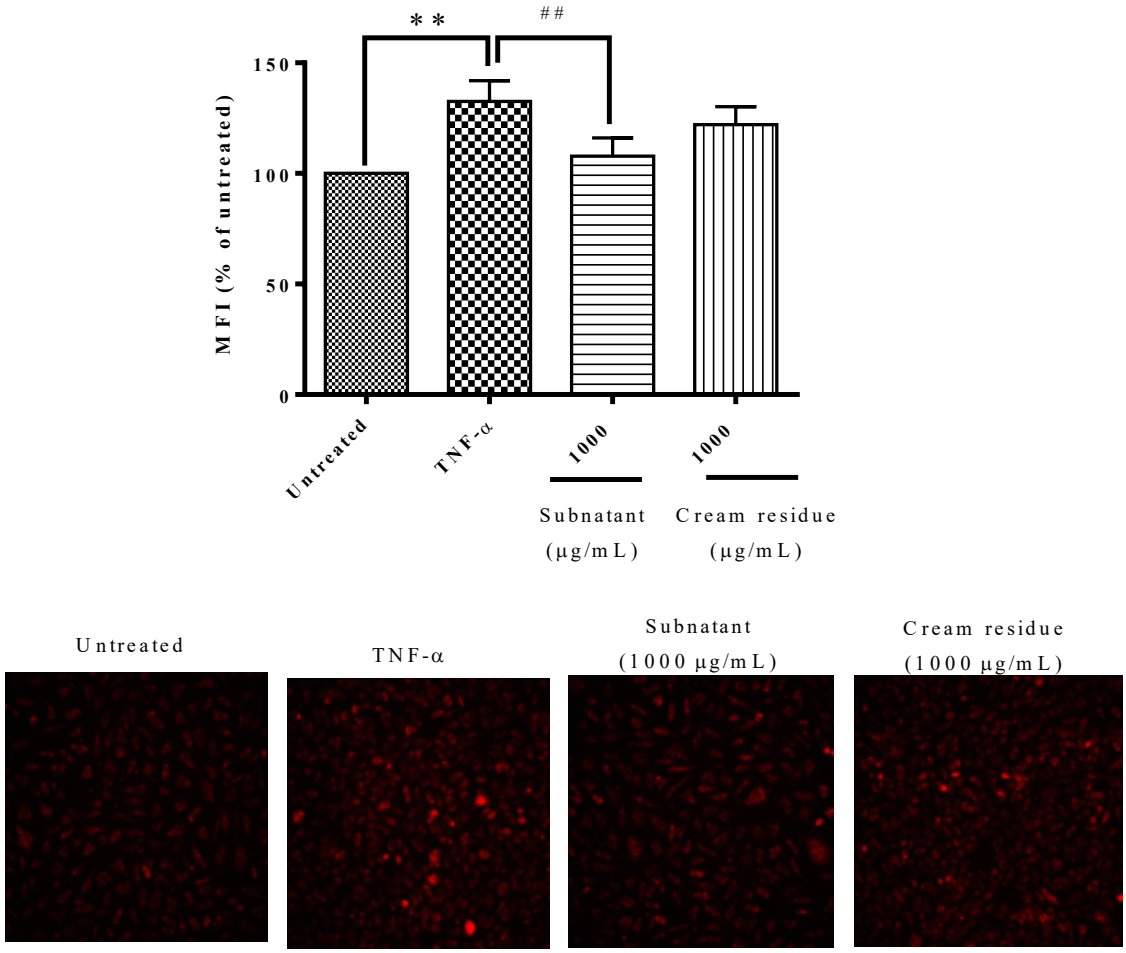

Figure 4.6. Effect of the subnatant and cream residue at the fixed concentration of 1000 μg/mL on TNF-α stimulated oxidative stress in endothelial cells. EA.hy926 cells were treated with 50 μM of different concentrations of the subnatant. Cells were treated with 10 μM of DHE for 30 min and the fluorescence signal was detected by fluorescence microscopy. Mean fluorescence intensity (MFI) was calculated based on the cell number and the data for each image. The data were shown as % of the untreated group. Data are presented as mean ± standard deviation based on 4 independent experiments. ** indicates $p < 0.01$ as compared to the untreated group, ## indicates $p < 0.01$ as compared to the TNF-α treated group.

4.4. Discussion

The subnatant was to be found effective in terms of the suppression of superoxide production at certain levels for endothelial cells and VSMCs. This could be explained by the compositional difference between the subnatant and the cream residue. Smaller protein fractions were observed in the subnatant compared to that for the cream residue (Fig. 3.9). The largest protein fraction found in the subnatant phase was about 69 kDa, whereas it was 110 kDa for the cream residue.

Peptides with small molecular weight potentially show bioactivity especially those between 2 kDa and 20 kDa (Martinez-Villaluenga and others 2017). On the other hand, 7.9% LPC (lysophosphatidylcholine, wt% of total lipids) was present in the cream before SC-CO_2 extraction while LPC was not detected in the egg yolk (Table 3.1). After SC-CO_2 + ethanol extraction, most of PL (93% recovery based on the feed material) was removed; however, some of the LPC still remained in the cream residue. LPC induces proinflammatory cytokines and oxidized LPC has both proinflammatory and cytotoxic properties (Huang and others 1999). Therefore, residual LPC in the cream residue is not expected to have antioxidant effect on the endothelial cells and VSMCs.

Blood vessels are comprised of endothelial cells and vascular smooth muscle cells (VSMCs). Endothelial cell line is a thin layer on the inner surface of blood vessels. Endothelial cells, which are in direct contact with blood, play an important role in vascular biology such as blood vessel tone, homeostasis, hormone trafficking and fluid filtration (El-Edela and others 2017). Vascular smooth muscle cells are within and compose the majority of the wall of blood vessels, which are not in direct contact with blood. Plaque formation between endothelial cells and VSMCs and vascular inflammation lead to increased blood pressure and risk of atherosclerosis. Furthermore, vascular inflammation and oxidative stress are key factors of endothelial dysfunction (Majumder and others 2013). Tumor necrosis factor (TNF), a pro-inflammatory cytokine, participates in the inflammatory response and progress of atherosclerotic lesions (Sprague and Khalil 2009). TNF triggers the generation of superoxide by activation of NADPH oxidase (Pennathur and Heinecke 2007). An increase in the superoxide level could cause impaired bioavailability of nitric oxide, endothelial vasodilator dysfunction and hypertension (Muller and Morawietz 2009). In conclusion, oxidative stress and inflammatory responses are interrelated. For this reason, an attempt was made to investigate the anti-inflammatory activity of the cream residue and the subnatant on both

endothelial cells and VSMCs. However, the results of Western blot analysis were not consistent, and more research is needed in this regard.

On the other hand, previously, Meram and Wu (2017) studied the effects of egg yolk livetins and their hydrolysates, prepared by proteolytic hydrolysis, on the inflammatory responses in lipopolysaccharide (LPS) stimulated murine RAW 267.7 macrophage in an *in vitro* model. Production of some inflammatory mediators such as nitric oxide (NO), prostaglandin-E2 (PGE2), pro-inflammatory cytokines, including tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) was observed. Dysregulated activation of some inflammatory enzymes, like iNOS and COX-2 were analyzed to understand the anti-inflammatory activity of livetins and their hydrolysates. In this study, the effect of egg yolk protein hydrolysates on inflammatory responses was studied following the protocols of Meram and Wu (2017). Ultrafiltration was used to separate the hydrolysate fractions with a molecular weight below 1 kDa from the subnatant and the cream residue. Production of some inflammatory mediators such as NO and dysregulated activation of inflammatory enzymes iNOS and COX-2 were evaluated to understand the potential anti-inflammatory activity of proteins and their hydrolysates in an *in vitro* model using LPS-stimulated RAW 267.7 macrophage cell line. Expression of iNOS and COX-2 was detected by Western blot analysis. The nitrite was quantified by Griess diazotization reaction according to the manufacturer's instructions (Molecular Probes, Inc., Eugene, OR, USA). However, the endotoxin levels of the samples were found to be higher than the limits (0.5 EU/ml) set by the US FDA (US Food and Drug Administration) after the ultrafiltration step. Removal of endotoxin from the samples was attempted using an endotoxin-free column (L00402, GenScript Inc., Piscataway, NJ, USA). However, the results of NO production and the expression of iNOS and COX-2 were not

consistent and it is recommended to repeat these experiments to have more consistent and conclusive results.

4.5. Conclusions

After the enzymatic hydrolysis and SC-CO_2 extraction, small protein fractions were observed in the subnatant and the cream residue, which could be biologically active. Cellular superoxide generation was detected by DHE and the cells were visualized by fluorescence microscopy. The effect of the cream residue and the subnatant on Ang II and TNF-α stimulated oxidative stress was examined in both VSMCs cells and endothelial cells. The results showed that the application of different concentrations (from 250 to 1000 μg/mL) of samples in the cell viability experiments did not exhibit any cytotoxic effects on VSMCs and endothelial cells. The study demonstrated that the subnatant at the concentration level of 1000 μg/mL showed an antioxidant effect in endothelial cells against TNF-α by significant ($p < 0.05$) suppression of the ROS-mediated fluorescence. The concentration levels (1000 and 500 μg/mL) of subnatant also showed an antioxidant effect in VSMCs against Ang II. However, the cream residue did not show a significant effect ($p > 0.05$) on the superoxide level in VSMCs and endothelial cells.

CHAPTER 5. Conclusions and recommendations

Egg yolk fractionation by 10-fold water dilution could be a good approach for IgY separation from egg yolk since 90% of total egg yolk lipids were kept in the leftover pellet (Kwan and others 1991). Fractionation and characterization of the valuable compounds in the pellet could help to utilize egg yolk lipids. Egg yolk lipids are composed of 62% triglycerides, 33% phospholipids (PL), and about 5% cholesterol (Anton 2007a). Phospholipids have broad applications in pharmaceutical and nutraceutical products, especially in infant formulas. Cholesterol has been used in nutraceutical and pharmaceutical products. On the other hand, cholesterol is no longer a concern of consumption because of the lack of evidence showing a relationship between cholesterol consumption and blood cholesterol levels (Dietary Guidelines Advisory Committee 2015; Ravnskov and others 2018). Supercritical CO_2 (SC-CO_2) has been used for the extraction of several lipids from different sources as a green solvent (Temelli 2009). The overall objective of this thesis research was to fractionate the valuable compounds in egg yolk and to characterize the fractions obtained for potential value-added applications. Therefore, the effect of enzymatic hydrolysis and pellet preparation on extractability of lipids such as cholesterol and phospholipids were studied in the first study (Chapter 3). The aim was to determine the antioxidant activity of egg yolk hydrolysates obtained by enzymatic treatment and the two-step SC-CO_2 extraction process in induced vascular muscle cells (A7r5) and endothelial cells (EA.hy926) in the second study (Chapter 4).

In the first study, a combination of Protease M and Lipase AY-30 treatment of egg yolk pellet facilitated the formation of a cream layer (Chapter 3). The cream had a higher lipid content (82%) compared to egg yolk (63%). Lipids were extracted using SC-CO_2 in two steps at 48.3 MPa, 70 °C and CO_2 flow rate of 1.0 L/min (measured at ambient conditions). In the first step, neutral lipids including cholesterol were extracted using neat CO_2. Polar lipids were extracted in the second step

with the addition of 8% (mole %) ethanol as co-solvent into SC-CO_2. Total extract yield (neutral lipids, cholesterol and phospholipids) from cream was higher than that from egg yolk. Hydrolyzing the pellet with Protease M and Lipase AY30 improved the extractability of cholesterol and PL. The recoveries of cholesterol and PL were 98% and 93%, respectively, based on the extraction feed material. Fatty acid profile analysis of the extracts from the second step showed the presence of higher levels of docosahexaenoic acid (DHA) and arachidonic acid (AA) in the polar lipid fraction. Microstructure analysis results showed the presence of dissociated individual particles (granules) in the residue samples after SC-CO_2 extraction. On the other hand, the increase in the purity of cholesterol (7.4%) in the extract obtained from cream was not significant compared to that from dry yolk (6%). The cholesterol purity was low for all samples (egg yolk, pellet and cream) because cholesterol was extracted together with neutral lipids in the first step of neat SC-CO_2 extraction. The improvement of the purities needs further investigation. The first step of SC-CO_2 extraction could be cascaded with different process conditions (pressure and temperature combinations) to extract high amount of cholesterol and neutral lipids in different steps. Detailed analyses, including cholesterol extraction kinetics have not been reported previously. A large proportion of cholesterol was obtained at the beginning of the extraction period using neat SC-CO_2 during the first 30 min. This information could explain the advantages of SC-CO_2 extraction process in terms of supplying energy and cost savings for scaling up purposes. The extraction kinetics of the other classes of egg yolk lipids such as phospholipids could be studied in the future. As well, quantification of the amount of free fatty acids formed due to lipase hydrolysis would be valuable to better assess the extent of hydrolysis and to design further fractionation approaches using SC-CO_2.

In the second study, the residue cream sample after SC-CO_2 extraction, and the subnatant phase after hydrolysis were shown to be rich in small molecular weight peptides, which could have biological activities such as antioxidant activity (Chapter 4). Cellular superoxide generation was detected by dihydroethidium (DHE) to determine the antioxidant activity of egg yolk proteins of the cream residue and the subnatant. The effect of the cream residue and the subnatant on Ang II and TNF-α stimulated oxidative stress was examined in both VSMCs and endothelial cells. The cell viability results showed that the application of different concentrations (from 250 to 1000 μg/mL) of samples did not exhibit any cytotoxic effects on VSMCs and endothelial cells. The study demonstrated that the subnatant had an antioxidant effect in VSMCs and endothelial cells against TNF-α by significant ($p < 0.05$) suppression of the ROS-mediated fluorescence. However, the cream residue did not show a significant effect ($p > 0.05$) on the superoxide level in VSMCs and endothelial cells. In this study, crude samples were used. Fractionation and further identification of the peptides present in the fractions with antioxidant activity needs further investigation. Antioxidant, anti-inflammatory and ACE-inhibitory activities are interrelated (Jahandideh and others 2015). After fractionation of peptides, other activities could be evaluated in future work.

Overall, this thesis research and findings contribute to the egg producers, processors and the food industry by providing approaches to utilize valuable components in egg yolk such as cholesterol, phospholipids and hydrolysates/proteins with antioxidant activity. In this research, SC-CO_2, which is a green solvent, was also used to extract cholesterol and phospholipids. This also meets recent demands for more eco-friendly processes for extraction rather than using harmful organic solvents.

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