DEVELOPMENT OF A FRACTIONATION PROCESS FOR THE PREPARATION OF A FOLATE-ENRICHED PROTEIN EXTRACT FROM HEN EGG YOLKS

Thèse

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RÉSUMÉ

Le fractionnement du jaune d'œuf est une façon judicieuse d'étendre les domaines d'application de cet ingrédient dans les industries alimentaire et nutraceutique. Le but de ce projet a été de mettre au point un fractionnement non-toxique du jaune d'œuf, par centrifugation, dans l'objectif d'obtenir un produit enrichi en extrait naturel de folate. De par sa teneur élevée en cholestérol, le jaune d'œuf a été considéré comme un sous-produit du procédé de séparation des œufs. Cependant, le jaune d'œuf contient aussi une forme précieuse et biodisponible d'acide folique. La dilution du jaune d'œuf et son fractionnement en granules et plasma par une technique de centrifugation (centrifugeuse de laboratoire et à échelle pilote) a permis l'obtention de granules d'une richesse en folate trois fois plus importante que celle du jaune d'œuf natif. Aussi, les granules obtenus ont présenté une concentration protéique deux fois plus élevée que celle du jaune d'œuf natif, et une concentration de lipides et cholestérol trois fois inférieure à celle du jaune d'œuf natif. Les granules sont apparus non solubles et de structure très compacte. Afin d'augmenter encore la concentration en folate, nous avons tenté de remettre les granules en suspension et de les séparer par centrifugation en utilisant des prétraitements tels qu'augmentation de la force ionique et traitements mécaniques (ultrasons puissants et pression hydrostatique élevée). L'application d'ultrasons puissants et l'augmentation de la force ionique n'ont que peu amélioré la concentration en folate. Les granules soumis à une force ionique de 0.15 M de NaCl et un traitement par ultrasons puissants de 10 minutes ont présenté des concentrations de 21 μg de folate / g de granulés. Une augmentation de la force ionique au delà de 0.15 M de NaCl a conduit à une concentration plus faible en folate, du fait de la déformation de la structure des granules et de la séparation de leur fraction soluble. Les observations ont indiqué qu'il pourrait y avoir une association entre la structure protéique des granules et leur contenu en folate. Les variations de solubilité et la modification du réseau structurel des granules par augmentation de la force ionique ont affecté la teneur en folate des structures granulaires. Les granules ont cependant présenté une structure difficilement modifiable sous ultrasons puissants et après augmentation de la force ionique. Le traitement à pression hydrostatique élevée (HHP), technique puissante, a été utilisé pour étudier l'effet des hautes pressions sur la concentration en folate des granules. Après 5 minutes de

traitement à 600 MPa, la concentration en folate a été mesurée dans les granules et le plasma, séparé des granules par centrifugation. Le plasma issu des granules contenait des concentrations en folate plus importantes que celles des granules précipités. Une analyse SDS-PAGE a permis de vérifier le profil protéique des granules sous l'effet du traitement HHP. Il est intéressant de noter que pour le plasma séparé des granules après traitement à haute pression, les migrations sur gel SDS-PAGE présentaient une bande protéique principale correspondant à la phosvitine. Les résultats de notre étude nous ont encouragés à proposer un modèle schématique de la structure des granules. Ces granules contiennent des HDL, LDL et phosvitine, et conservent le contenu total en folate du jaune d'œuf. Les protéines des granules sont principalement phosphorylées et il existe une forte liaison entre les apoprotéines de HDL et la phosvitine du fait de ponts de phosphate de calcium. Des traitements mécaniques ont libérés des particules de LDL qui pourraient être piégées dans le réseau structurel des granules. Les interconnexions entre les apoprotéines de HDL, la phosvitine et le folate pourraient se faire par des ions calcium. Nos résultats mettent en évidence le potentiel du procédé afin de produire un concentré riche en folate à partir des jaune d'œuf. Cependant, d'autres travaux seront nécessaires afin de trouver des utilisations aux co-produits (plasma) et rendre le procédé viable à l'échelle commerciale.

ABSTRACT

Fractionation of egg yolk is a smart way to expand the application of egg yolk ingredient in food and nutraceutical industry. The goal of this project was to develop non-toxic fractionation process of egg yolk by using centrifugation in order to prepare a natural folate-enriched extract. The egg yolk has been considered as a by-product of egg separation process due to its high cholesterol content. However, egg yolk contains valuable and bioavailable form of folate. Dilution of egg yolk and its fractionation into granule and plasma by centrifugation technique (lab- and pilot-scale centrifuge) resulted in separation of a granule fraction being rich in folate which was 3 fold higher than native egg yolk. This granule fraction was also characterized by high protein concentration (2 fold higher than protein content of yolk) and lower lipid and cholesterol (3 fold) content compared to non-treated egg yolk. The granule fraction appeared to be non-soluble with a very compact structure. By using the pre-treatments techniques such as increasing ionic strength and mechanical treatments (ultrasound and high hydrostatic processes), we attempted to re-suspend granules and separate them by centrifugation in order to further increase folate concentration. Results demonstrated that ultrasound and increased ionic strength did not largely change folate concentration. At ionic strength 0.15 M NaCl and after 10 min of ultrasound treatment granule contained 21 μg folate/g granules. By increasing ionic strength higher than 0.15 M NaCl the folate concentration was lower in granule due to the disruption of granule structure and separation of soluble fraction of granule. The observations denoted that there might be association between granular protein structure and folate content. Changes in solubility and disruption of granule network structure by increasing ionic strength affected folate content of granule structure. However, granules appeared to have very stable structure under the ultrasound and after increasing ionic strength, and their modifications were not easily possible. The high hydrostatic pressure processing (HHP) was used as an innovative and powerful technique in order to study the effect of high and drastic pressure on the concentration of folate in granules. After 5 min of 600 MPa HHP treatments, the folate concentration was measured in granule and separated plasma from granule after centrifugation. Plasma from granule contained higher concentration of folate compared to the precipitated granule. SDS-PAGE analysis was used

in order to verify the granule protein profiles as a function of HHP treatment. Interestingly, the plasma separated from granule after HHP treatment contained phosvitin as a leading protein band separate in SDS-PAGE gel. The results of our study allowed proposing a schematic model for the granule structure which contains HDLs, phosvitin and LDLs. Beside; granule contains large amount of folate. Proteins of granule are mostly phosphorylated and strong connection between apoproteins of HDLs and phosvitin exists through calcium phosphate bridges. LDL particles were liberated through mechanical treatments and might be entrapped in the granular network. The interconnection between the apoproteins of HDLs, phosvitin and folate could be through calcium ions. Our results provided highly promising evidences concerning the recovery of high-concentration folate extract from hen egg yolk. Our fractionation technique is also clean but it generates plasma as co-product that is still usable in food formulation. Such applications still need to be developed before the technology can be viable at commercial scale.

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ABREVIATIONS

5-MTHF: 5-methyltetrahydrofolate

CLSM: Confocal laser scanning microscope

DHA: Docosahexaenoic acid

DRI: Dietary reference intake

FA: folic acid

GPC: Gel-permeation chromatography

HDL: High density lipoproteins

IEC: Ion exchange chromatography

IgY: Immunoglobulin Y

IOM: Institute of Medicine

IUPAC-IUB: International Union of Pure and Applied Chemistry-International Union of Biochemistry

LDL: Low density lipoproteins

MUFA: Monounsaturated fatty acids

NTD: Neural tube defects

PC: Phosphatidylcholine

PE: Phosphatidylethanolamines

PI: Phosphatidylinositol

PS: Phosphatidylserine

PUFA: Polyunsaturated fatty acids

SDS-PADE: sodium dodecyl polyacrylamide gel electrophoresis

SFA: Saturated fatty acids

SM: Sphingomyelin

UFA: Unsaturated fatty acids

VLDL: Very-low-density lipoprotein

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A great many people have contributed irreplaceable to this thesis and because of whom my graduate experience has been one that I will treasure forever. I therefore wish to greatly acknowledge:

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FOREWORD

The work carried out in this thesis was aimed to develop egg yolk fractionation process to prepare a natural-folate enriched extract. This thesis contains all the results obtained during the course of realization of the project. Results are presented in the form of 3 research articles.

The first article "Scaling-up a process for the preparation of folate-enriched protein extracts from hen egg yolks" has been submitted and published in "Journal of Food Engineering" (2014, 141: 85-92). Authors are Nassim Naderi, James D. House and Yves Pouliot.

The second article "Effect of selected pretreatments to increase the folate content of granule suspensions prepared from hen egg yolk" is in preparation for a submission in "Journal of Food Engineering". Authors are Nassim Naderi, James D. House and Yves Pouliot.

The third article "Understanding the effect of ionic strength and mechanical treatments on the composition and microstructure of granule separated from hen egg yolk" is in preparation for a submission in "Journal of Agriculture and Food Chemistry". Authors are Nassim Naderi, Alain Doyen, James D. House and Yves Pouliot.

For each article, Nassim Naderi[1] planned and achieved the experiments, presented the results and wrote articles. In consequence, she is the first author of all articles. Dr. James D. House[1,2] is co-supervisor of this project and he participated to the planning, discussion of results and the revision of the writing of all articles. Dr. Alain Doyen[1] is second co-author of third article and he participated in discussion of results and revision of article. Dr. Yves Pouliot[1] is supervisor of this project and he participated to the planning, the discussion of the results and the revision of the writing of all articles. He is the last author of all articles.

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To who loves folate and the people who lived with Spina bifida!

Life consists in penetrating the unknown, and fashioning our actions in accord with the new knowledge thus acquired. ~Leo Tolstoy

It may not be possible to win the future but if we're going to encourage more innovation, it's not enough for us to just dig in and work harder. We also need to encourage surprise and serendipity.
We need to play each other's instruments
~ Steven Johnson

CHAPTER 1: INTRODUCTION

The importance and consumption of the eggs by human dated back to antiquity. Eggs are cheapest source of animal protein that is used throughout the world.

The worldwide consumption of egg over the past twenty years has faced two different trends. The egg consumption declined by 14% until the mid-1990s. Since that time, the consumption of eggs has remained fairly constant. The decline of egg consumption by consumers in the first part of this period was partially due to concerns about eggs contributing to cholesterol levels. The other factors that affected consumption of egg were the lack of convenient egg products on the market. Therefore, the research and development of new processes and new value-added egg products will expand egg industry and add more benefit compare to simple processing for the egg breaking industry (Sim and Nakai, 1994).

Eggs are largely composed of the protein and lipids. Protein fraction is highly digestible and contains the most important essential amino acids in a profile that is not dissimilar to the ideal balance of amino acids needed by men and women. Egg yolk lipids are composed of triglycerides, phospholipids, cholesterol and free fatty acids. Based on the diet of hen the fatty acid composition of yolk corresponds to saturated fatty acids (SFA), polyunsaturated fatty acids (PUFA) and monounsaturated fatty acids (MUFA). Some of yolk lipids are particularly suitable for nutrition or health such as omega 3 fatty acids. Furthermore, eggs contain major vitamins and minerals. Some of those nutrients (i.e. folate, vitamin E, vitamin D, manganese, selenium) in egg can be manipulated by dietary means in order to improve the human diet (Surai and Sparks, 2001). Eggs are considered as an excellent source of folate and research showed that increasing the folate content of eggs will position the egg as an important source of dietary folate (Dickson et al., 2010; Hebert et al., 2005).

Folate is essential water soluble B vitamin that acts as cofactors and co-substrates for different biological methylation reactions such as those involved in amino acid and nucleic acid synthesis. However, humans are devoid of folate biosynthesis, therefore, they are

completely dependent on dietary sources to meet their daily folate requirements. Sufficient folate status in humans is reported to lower the risk of neural tube defects (NTD) in babies (Czeizel and Dudas, 1992). Folic acid is a synthetic form of folate and is the major supplemental form used in food fortification. In the late 1990s, the governments of Canada and the USA introduced the mandatory fortification of folic acid in cereal grain products to reduce the number of NTD occurrences in newborn babies. However, while cases of NTDs were reduced significantly, an emerging body of literature suggests that consuming high levels of folic acid may have several negative consequences. As a result, attention has been directed into other strategies that may help to increase the population's intake of natural folates.

Eggs naturally contain about 22 μg of folate (USDA, 2001), which is equivalent to 6% of the established adult daily requirements for folate. Based on recent research studies, synthetic folic acid added to laying hen diets was deposited in the egg yolk as natural folate (House et al., 2002; Sherwood et al., 1993) with high bioavailability. One large folate-enriched egg provides approximately 45 to 50 μg of folate, or roughly 12% of the current recommended nutrient intakes for adults that is 400 μg/day (DRIs, 1998). However, because one large egg contains about 210 mg of cholesterol, public has been recommended to limit their egg consumption (Krauss et al., 2000).

The current fractionation techniques available at industrial scales have enabled the development of bioactive-enriched food derived extracts. Since egg is widely used for its nutritional and functional properties, it is necessary to develop processes for the production of folate-enriched extract from egg yolk in order to improve health benefits of egg base products.

The goal of this work was to develop a non-toxic egg yolk fractionation process which is easily scalable for industrial application at the prospect of produce a proteinaceous extract from egg yolk enrich with natural folate with lower concentration of cholesterol.

CHAPTER 2: LITERATURE REVIEW

2.1. HEN EGG YOLK

Eggs produced for human consumption are infertile from hens classified as *Gallus domesticus*. The ovary of female chick contains numerous (over 3000) small ova at the time of hatching. The egg yolk is formed in three steps within the final 10-12 days prior to laying of egg: (1) the part that is formed during embryonic development of the female chick, (2) the normal slow development of the ovum from the time of hatching of the chick to the point in sexual maturity (some ten days before ovulation), and (3) the accelerated growth period during the last ten days before ovulation (release of the ovum or yolk) into the oviduct, a part of the female reproductive system. The egg white (albumen) is secreted in oviduct to surround the yolk and finally shell and its membranes are deposit to complete the formation process (Stadelman et al., 1995).

The average weight of eggs produced by selectively hen bred strains is 60 g. About 10% of egg is composed of shell, 59% albumen and 31% yolk. From the edible part of egg, 74.4% is water, 12.3% protein and 11.6% lipids. Egg contains all vitamins except vitamin C (Seuss-Baum and Nau, 2011).

2.1.1. Overall composition and structure

Egg yolk material is surrounded by vitelline membranes. It prevents mixing of yolk and egg white (albumen) and forms the last barrier to microbial infection. A thin continuous layer is located between two fibrous layers, the inner layer facing the oocyte (formed in the ovary), and the outer layer facing the albumen (deposited in the oviduct).

A freshly laid yolk contains 50 to 52% dry matter based on the age of the laying hen and the length of storage. Egg yolk is mainly composed of lipids (about 65% on dry basis) with a lipid to protein ratio of about 2:1. The lipid composition of egg yolk is made up of 62% triglycerides, 33% phospholipids, 5% cholesterol and less than 1% carotenoids which give

it its color. Based on a standardized feed of hens, the fatty acid composition of lipids in egg yolk consist of 30–35% saturated fatty acids (SFA), 40–45% monounsaturated fatty acids (MUFA), and 20–25% polyunsaturated fatty acids (PUFA). Oleic acid (C18:1, 40-45%), palmitic acid (C16:0, 20-25%), and linoleic acid (C18:2, 15-20%) are the main fatty acids in egg yolk (Wang et al., 2000). However, the fatty acid composition of egg yolk is highly reflective of the diet of laying hen.

The triglyceride contains most of the total fatty acid. The glycerol of the triglycerides is generally esterified by palmitic acid in position 1, by oleic and linoleic acids in position 2, and by oleic, palmitic, and stearic acids in position 3 (Kuksis, 1992). Egg yolk also contains great amount of phospholipids. Phospholipids are amphiphilic molecules which contain one hydrophilic head group (phosphoric acid + alcohol and amino acids), and one hydrophobic group (two fatty acids). Phospholipids in egg yolk are mainly composed of phosphatidylcholine (PC) which is 76% of total phospholipids. From other phospholipids, yolk contains 22% phosphatidylethanolamine and very low amount of phosphatidylinositol (PI), phosphatidylserine (PS), and sphingomyelin (SM). Fatty acids in phospholipids composed of 45% saturated fatty acids (SFA), 30–40% polyunsaturated fatty acids (PUFA), and 20–25% monounsaturated fatty acids (Kuksis, 1992).

Egg yolk also contains 5% cholesterol. About 85–90% of cholesterol is in free form which involves in the structure of low density lipoproteins (LDLs) and 10-15% in esterified form. The cholesterol esters are located in the lipid core of LDLs which contains oleic (35%), palmitic (33%), linoleic (12%) and stearic acid (11%) (Anton, 2007a).

Proteins in egg yolk are present as free proteins or apoproteins which contribute in lipoprotein assemblies. Lipoproteins (low and high density lipoproteins) are main constituents of egg yolk that formed due to the interactions between lipids and proteins. The major egg yolk proteins components are 68% low density lipoproteins (LDL), 16% high density lipoproteins (HDL), 4% phosvitin, and 10% livetins (Mine, 2010).

Egg yolk is a complex system and it has been considered as a natural oil-in-water emulsion. It is a pseudoplastic non-Newtonian fluid with viscosity which depends on the shear forces applied. It has a pH around 6.0 that only slightly increases (pH 6.4-6.9) after prolonged

storage. Anton (2013) provided one of the latest description for the micro structure of egg yolk which consist of non-soluble protein aggregates (granules) in suspension in a clear yellow fluid (plasma) that contains LDLs and soluble proteins (Figure 2.1). Therefore, yolk can be separated into its two main fractions of plasma and granules by mild centrifugation, a technique largely used for protein analysis in both fractions. Figure 2.2 presents a schematic representation of fractionation of the egg yolk and major composition of each fraction. Since 1960s up to now, a wide range of centrifugation methods with different parameters (i.e. dilution agents and dilution factor, centrifugation speed and time, pre-treatments.) have been used to separate plasma and granule and investigate their lipoprotein structure (Anton and Gandemer, 1997; Burley and Cook, 1961; Cook and Martin, 1969a; Laca et al., 2010; Martin et al., 1964; Mc Bee and Cotterill, 1979; Strixner and Kulozik, 2013).

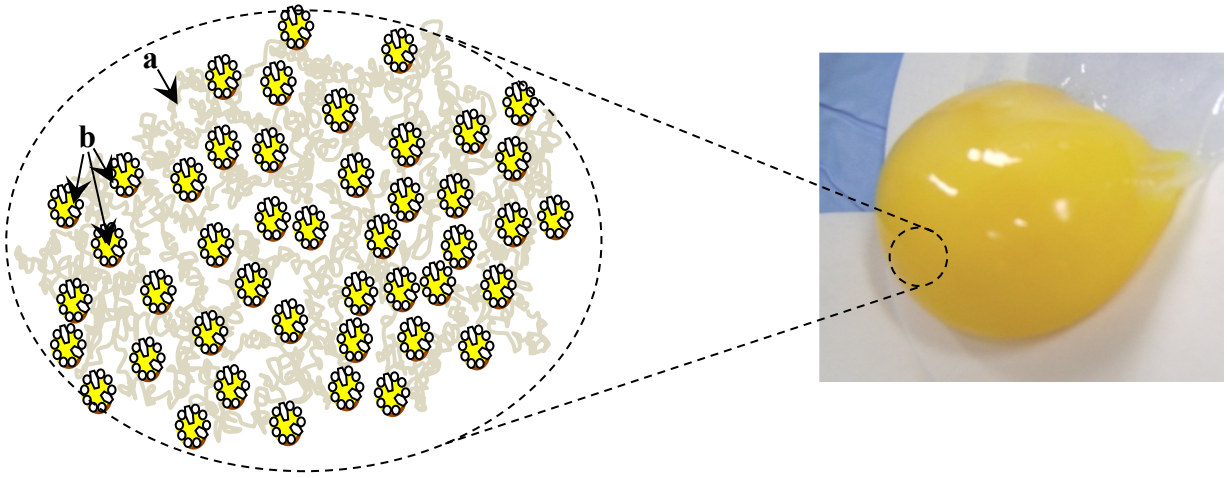


Figure 2.1. Components of the micro structure of egg yolk- a: Granule, b: LDL in plasma

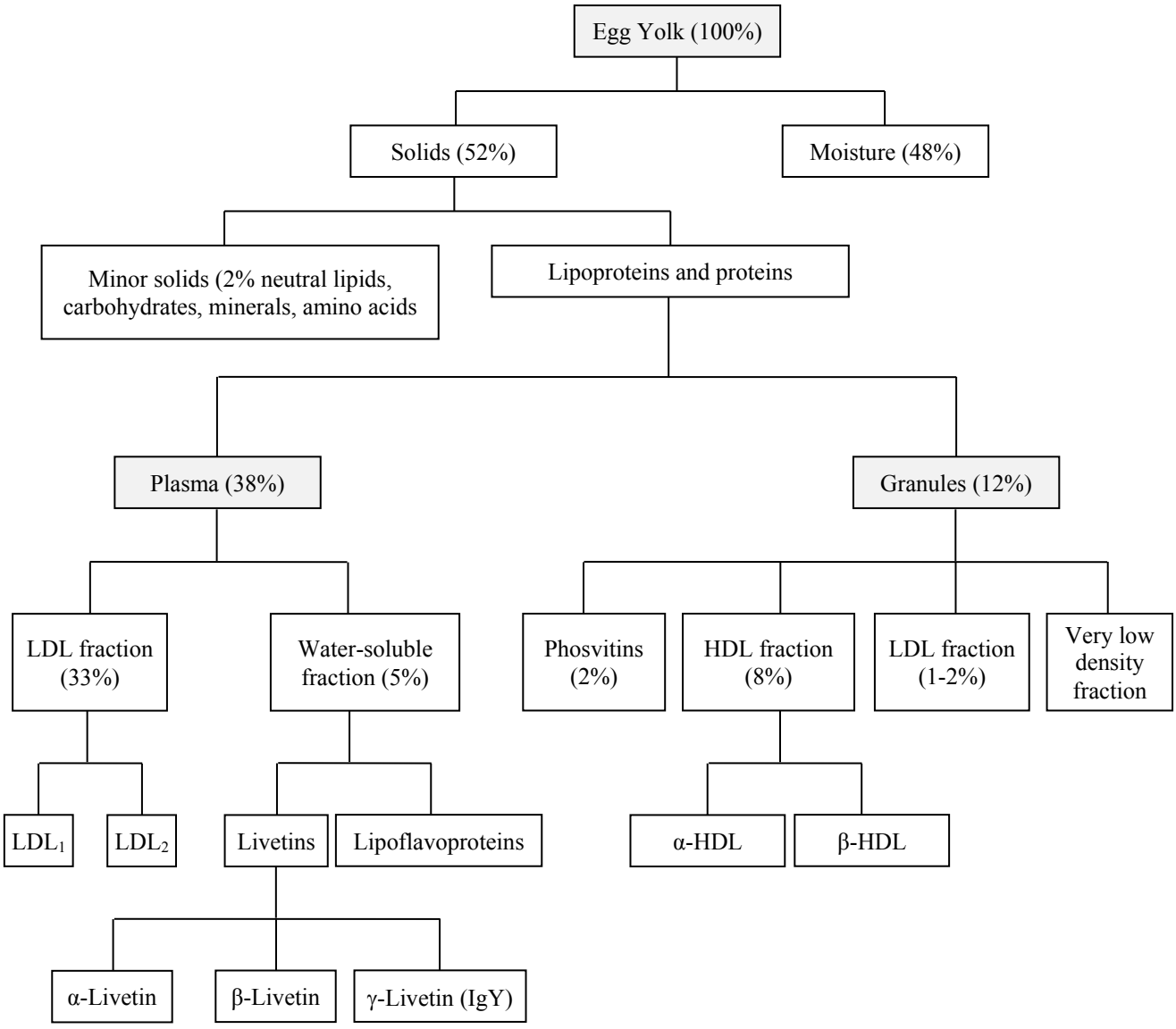


Figure 2.2. Schematic presentation of egg yolk fractions and their composition; Adapted from Li-Chan et al. (1995)**.**

The egg yolk particles have been classified based on their spherical sizes (4-150 μm in diameter), and granules (0.84-4.87 μm). The dilution and centrifugation of egg yolk result into two distinct factions: plasma, a dark orange supernatant fraction and granule, a precipitated pellet fraction with pale color. The granules contain 12% LDL but mainly consist of HDLs (60%) and phosvitin (16%) linked together through phosphocalcic bridges. Due to the high protein content, granules have a density of 1.190 g/ml. The plasma is extremely rich in LDLs (85%) with 15% livetins (Anton, 2007a). The density of LDL micelles is reported between 0.908 g/ml and 1.063 g/ml (Hevonoja et al., 2000; Li-Chan et al., 1995). The low density of LDLs has been attributed to their much higher lipid:protein content ratio which makes them more soluble in aqueous solution (Anton, 2007a; Anton et al., 2003).

The micro structure of egg yolk has been investigated in several studies. In earlier works, the transmission electron microscopy has been used to investigate the structure of particles in egg yolk (Chang et al., 1977). In their micrographs, the HDLs were presented as high electron-dense microparticles which were seemed to be attached to hair like strands (phosvitin molecules) even in 1.71M NaCl. On the surface of plasma LDLs, numerous electron-dense particles were observed. In another study, the researchers used transmission electron microscope to study the microstructure of fresh egg yolk. In their observations histological differences were observed in the yolk spheres in each yolk layer. The yolk spheres were closely packed in the vitelline membrane, with various sizes and shapes. The protein granules with high electron density were observed in the yolk sphere and granules were distributed between the outer and inner layers in different amount and with different shape (Kobayashi, 1997).

LDLs (lipovitellenins) micelles are spherical particles of about 20–60 nm diameter with a neutral lipid core (triglycerides and cholesterol esters) surrounded by apoproteins and phospholipids. About six apoproteins have been reported in LDL structure which was characterized by their high hydrophobicity and flexibility. These apoproteins, named apovitellenins I-VI, represent about two third of yolk solids (Li-Chan and Kim, 2008). Very-low-density lipoproteins (VLDL), the precursors of LDL micelles, have a size range of 80 to 350 nm and are detectable in the plasma fraction of egg yolk even after transferring

some egg yolk compounds (Huopalahti et al., 2007; Sirvente et al., 2007). The plasma fraction of yolk is mainly composed of 85% LDL and 15% livetin. Livetins are water soluble globular proteins which are composed of α-livetin (serum albumin), β-livetin (α_2-glycoprotein), and γ-livetin (γ-globulin/immunoglobulin Y: IgY) (Li-Chan and Kim, 2008).

Unlike LDLs, high density lipoproteins (HDL) or lipovitellin structure is similar to globular proteins. In HDL, the protein can be loaded with varying amount of non-covalently bound lipid from which phospholipid holds two third of lipids. Vitellogenin is the precursor of HDLs and it is synthesized in the liver and loaded with phosphorylated, glycosylated lipids and there it interacts with at least two Ca^{2+} ions and one Zn^{2+} ion (Anderson et al., 1998). HDLs, which represent 70% of the granule's composition, consist of α- and β-HDL which are different in terms of amino acid composition, particle-bound phosphorus and carbohydrate content. Moreover, α-HDL has more acidic nature compare to β-HDL due to its higher sialic acid content. About five apoproteins have been recognized in the HDL structure (Guerrero-Legarreta, 2010). Phospholipid is the principal component in HDL while triglycerides and other neutral lipids make up the majority of the lipid in LDL and VLDL. The lipid contained in the HDL is bound in a noncovalent manner and is extractable with organic solvents (Anderson et al., 1998). The HDLs are structured through five disulfide bridges as well as several ionic and hydrophobic interactions. HDLs make complexes with phosvitin through phosphocalcic bridges which is a basic element of egg yolk granules.

At low ionic strength the HDL-phosvitin form non-soluble complexes which cause the granule structure to be very compact and weekly accessible to enzymatic digestion and also resistant to thermal denaturation or even heat gelation (Anton, 2007a). In the granules, HDLs are organized in a multilevel structure (Strixner et al., 2014) as presented in Figure 2.3. The smallest parts are the HDL proteins with a molecular weight of about 400 kDa and diameters between 7 and 20 nm (Burley and Cook, 1961).

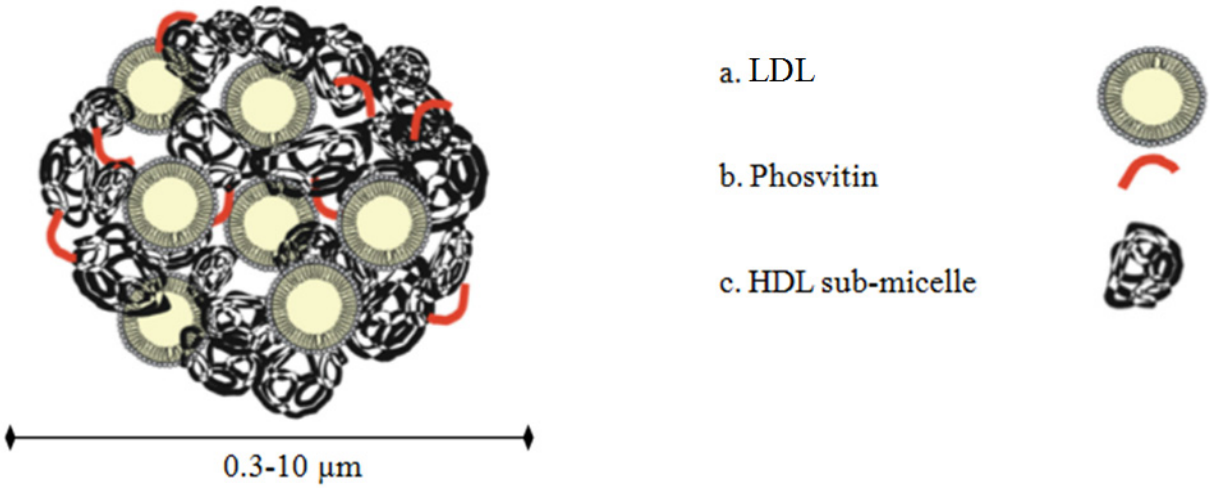


Figure 2.3. Schematic model proposed for egg yolk granule at pH 6.5. HDL-submicelles (c) linked to phosvitin (b) through calcium phosphate bridges and embedded LDL vesicles (a). Adapted from Strixner et al. (2014)**.**

The atomic force microscopy studies indicated the pH-dependent modification of HDL-granule structure. At pH 4.0, LDLs were strongly associated to the HDL-granules. LDLs were clearly identified based on their spherical shape and smooth surface. At pH 6.5, HDL granules form a protein network and the HDL-subunits were formed by loop like coiled strands (Strixner et al., 2014).

The electron microscopy images of native granules also revealed a satin-like rough surface for granules which were closely packed and intact with average size between 0.5 and 2 µm in diameter (Bäckermann et al., 2008). In earlier investigations of Chang et al. (1977), the granular structure consists in circular complexes ranging in diameter from 0.3–2 µm. In their recent investigations, Strixner et al. (2014) used different centrifugal force and determined that the granule size was ranged 0.8 to10 µm. However, there is still a gap of knowledge concerning the molecular structure of egg yolk HDL granule under different process conditions (i.e. in presence of salt, mechanical treatments) and further work is necessary to establish a link between the nano structure and composition of granules from hen egg yolks (Strixner et al., 2014).

Phosvitin, a phosphoglycoprotein, represents 11% of total egg yolk protein and 16% of granule proteins. Phosvitin is a glycoprotein with an exceptionally high amount of phosphoric acid bound to serine residues. Phosvitin consists of two polypeptides (α- and β-phosvitin). Alpha-phosvitin (160 kDa) contains three to four subunits of 35-40 kDa and β-phosvitin (190 KDa) contains four to five subunits of 45 kDa (Anton et al., 2007) . Amino acids of these subunits consist of about 50% serine. There is no or little sulfur containing amino acid in their composition (Anton et al., 2007). Phosvitin is extremely hydrophilic, and it has numerous negative charges and unusually low percentage of nonpolar hydrophobic side chains (Dickinson et al., 1997) with isoelectric point of pH 4.0 (Ternes, 1989). Phosvitins are highly phosphorylated proteins and strongest metal-binding biomolecules found in nature. Indeed, about 95% of iron in egg yolk is bound to phosvitin and more than 50% of its residues are serine of which more than 90% are phosphorylated. Similarly to HDLs, phosvitin is synthesized from vitellogenin but the two polypeptides (phosvitin and HDL) are liberated after cleavage of vitellogenin and interact through phosphocalcic bridges to form granular structure (Anton, 2007a).

Thermal (90ºC) or high pressure methods (600 MPa) did not resulted in aggregation and stronger or weaker iron binding capacity of phosvitin compared to that of untreated. These results indicated that unordered and high negatively charged structure of phosvitin prevents unfolding or modification by heat or pressure treatment (Castellani et al., 2005).

Minerals account for 1% of the egg yolk content. The major mineral in yolk is phosphorus which is primarily in bound form in phospholipids. Other minerals such as calcium, chloride, potassium, sodium, sulfur, magnesium and manganese are represented in lesser quantities and can be altered in egg yolk if hens diet is modified (Mine, 2010). Finally, higher vitamin concentration, represented by fat-soluble vitamins of A, D, E and also water-soluble vitamins such as folate, riboflavin, niacin and B12, was found in egg yolk compared to albumen.

2.1.2. Nutritional and nutraceutical properties of egg yolk

Eggs are excellent source of several important nutrients naming protein, monounsaturated fatty acids, polyunsaturated fatty acids, cholesterol, antioxidant carotenoids, choline, folate, iron, calcium, phosphorus, selenium, zinc and vitamins A, B2, B6, B12, D, E and K. Egg proteins have unique biological activities besides their excellent nutritional value with a good balance of essential amino acids (Hasler, 2000; Kerver et al., 2002; Watkins, 1995). Nutritional compositions of eggs (whole egg, egg yolk and egg white) are presented in Table 2.1. Nowadays, many research studies relating to utilization of the egg as a carrier of special nutrients or using the egg as the source of specific chemicals or pharmaceuticals.

Table 2.1. Composition of whole egg, yolk and white.

Composition (in 100 g)	Whole egg	Egg white	Egg yolk
Water (g)	74.4	88.6	49
Protein (g)	12.3	10.6	16.1
Lipid (g)	11.9	0.1	34.5
Triglycerides (g)	7.7	-	22.9
Phospholipids (g)	3.4	-	10.0
Cholesterol (g)	0.42	0	1.2
Lecithin (g)	2.3	0	1.2
Saturated fatty acids (g)	4.4	-	13.0
Palmitic acid (C16:1)	2.5	-	7.3
Stearic acid (C18:0)	0.86	-	2.5
Unsaturated fatty acids (g)	7	-	21.7
Palmitoleic acid (C16:1)	0.4	-	1.1
Oleic acid (C18:1)	4.1	12	-
Linoleic acid (C18:2)	1.25	-	3.6
Linolenic acid (C18:3 n-3)	0.04	-	0.12
Arachidonic acid (C20:4 n-6)	0.2	-	0.6
EPA (C20:5 n-3)	0	-	0
DHA (C22:6 n-3)	0.15	-	0.4
Essential amino acids (mg)			
Histidine	-	-	-
Isoleucine	290	240	410
Leucine	660	560	870
Lysine	1040	880	1390
Methionine+Cystine	820	660	1170
Phenylalanine+Tyrosine	640	670	660
Threonine	1150	1020	1420
Tryptophane	590	470	850
Valine	190	170	240
Carbohydrate (g)	0.7	0.8	0.5
Ash (g)	0.9	0.5	1.6
Sodium (mg)	120	155	50
Chlorine (mg)	172	175	162
Potassium (mg)	125	140	100
Calcium (mg)	50	8	133
Phosphorus (mg)	193	18	530
Iron (mg)	1.7	0.1	4.8
Magnesium (mg)	12	10	15
Sulphur (mg)	164	163	165
Zinc (mg)	1.4	0.12	3.9
Copper (mg)	0.06	0.02	0.14
Manganese (mg)	0.04	0.007	0.11
Iodine (mg)	0.05	0.003	0.14
Ascorbic acid (µg)	0	0	0
Vitamin A (µg)	150	0	450
Vitamin D (µg)	1.5	0	4.5
Vitamin E (µg)	1200	0	3600
Vitamin B1(µg)	913	10	250
Vitamin B2(µg)	447	430	480
Vitamin B6(µg)	133	10	370
Folate (µg)	56	12	140
Niacin (µg)	79	90	60
Biotin (µg)	25	7	60
Pantothenic acid (µg)	1700	250	4500

Adapted from: (Nys and Sauveur, 2004; Seuss-baum, 2007)

The egg yolk is a reservoir of antibodies (called IgY) with many proven uses as well as many theoretical applications. The main problem in isolating IgY from egg yolk is separating the lipoproteins from the egg yolk before purification of the IgY. Thereby, several purification methods of IgY have been reported, including lipoprotein separation by ultracentrifugation (Mc Bee and Cotterill, 1979), delipidation by organic solvents (Horikoshi et al., 1993), and lipoprotein precipitation by polyethylene glycol (Polson et al., 1980), sodium dextran sulfate (Jensenius et al., 1981), sodium alginate (Hatta et al., 1990), and ultrafiltration (Kim and Nakai, 1998, 1996). The use of egg antibodies (IgY) to prevent or treat gastrointestinal (GI) pathogens in both humans and animals is a recent and exciting application of egg technology (Mine and Kovacs-Nolan, 2002).

Lysozyme from egg is also used extensively as a food preservative. It shows high antimicrobial activities against mesophilic and thermophilic spore-forming bacteria. Consequently, lysozyme prevents the growth of pathogenic bacteria on refrigerated foods (Johnson, 1994). For example, this compound is used in cheese making process since it has no inhibitory effect on starter and secondary cultures required for the ripening of the cheeses but prevents contamination by spoilage and pathogenic bacteria.

The capacity of phosvitin from yolk for metal chelation makes it a very important natural food antioxidant. Phosvitin contains high proportion of phosphoserine residues responsible for its iron binding capacity. Some works demonstrated that iron fixation is pH and ionic strength dependent under the conditions employed. Indeed, at pH value of 3.5, the chelation of iron is inhibited by protonation of the phosphate groups and by conformational changes in the protein (Castellani et al., 2004). However, acidification of the solution after iron fixation does not promote the release of iron. This was explained by the presence of partially protonated phosphates that could inhibit the formation of iron/phosvitin complex. Thus, Castellani et al. (2004) have established that a pH value of 6.5 and ionic strength of 0.15 M NaCl represented the optimal conditions for iron fixation by phosvitin to an optimized concentration of 115 μg iron/mg protein. The antioxidant activity of phosvitin can be improved by chemical modification by conjugation with galactomannan through a controlled Maillard reaction at 60 °C in 79% relative humidity for one week. It was observed that the conjugation reaction significantly enhances the antioxidant activity of

phosvitin, and even improves emulsifying activity, emulsion, and heat stability (Nakamura et al., 1998).

Egg yolk lecithin contains great quantity of phosphatidylcholine. Phosphatidylcholine plays an important role in intestinal lipid absorption by enhancing micellar lipid solubility and formation of chylomicrons. Research showed that the intestinal absorption of egg cholesterol may be reduced by the presence of phosphatidylcholine and sphingomyelin from egg yolk (Jiang and Mine, 2001). Moreover, phosphatidylcholine is particularly valuable nutritionally because it is the source of two important nutrients of choline and polyunsaturated fatty acids (PUFA) from the n-3 and n-6 PL family. Consequently, interest for the production of phosphatidylcholine or lecithin from egg yolk has increased, especially if the production of polyunsaturated fatty acids enriched phospholipids may be increased through feeding modifications of laying hens (Gładkowski et al., 2012). A summary of egg components and corresponding biological activities are presented in Table 2.2.

Table 2.2. Egg compounds with summary of their biological activities

Applications	Egg compounds	References
Antibacterial activity	Lysozyme	(Baron and Rehault, 2007)
	Ovotransferrin	″
	Proteinase inhibitors: Ovostatin	″
	Serine protease inhibitors: Ovomucoid and ovoinhibitor	″
	cystatin inhibitors	″
	Vitamin-binding proteins: riboflavin-binding protein, avidin, thiamin-binding protein.	″
Antihypertensive Activity: Egg-Protein-Derived	Peptic digest of ovalbumin (sequence: ERKIKVYL)	(Iroyukifujita et al., 2000)
Peptides with	Peptic digest of ovalbumin (sequence: FFGRCVSP)	″
	Peptic digest of ovalbumin (sequence: LW)	″
	Peptic digest of ovalbumin (sequence: SALAM)	(Pellegrini et al., 2004)
	Peptic digest of ovalbumin (sequence: FRADHPFL)	(Fujita et al., 1995)
	Peptic digest of ovalbumin (sequence: RADHPFL)	(Miguel et al., 2004)
	Chymotryptic digest of ovalbumin (sequence: RADHPF)	(Matoba et al., 1999)
	Pancreatic digest of FRADHPFL, RADHPFL (sequence: RADHP)	(Miguel et al., 2006)
	Peptic digest of ovalbumin (sequence: YAEERYPIL)	(Davalos et al., 2004; Miguel et al., 2005)
	Pancreatic digest of YAEERYPIL (sequence: YPI)	(Davalos et al., 2004)
Immunoglobulin (IgY) antibodies in therapeutic or prophylactic applications	Gamma-Livetin (IgY): In veterinary medicine (treatment of intestinal infections, IgY application in aquafarming) In Human Medicine (treatment of intestinal infections in children, treatment of helicobacter pylori, use of IgY for treatment of colitis and celiac disease, treatment of cystic fibrosis, prophylactic use of IgY in dental caries, use of IgY for treatment of poisonings, in Proteomics)	(Huopalahti et al., 2007b)
Metal-binding capacity and biological activities	Ovotransferrin (Ion-binding capacity, metal-binding capacity and biological activities)	(Guérin-Dubiard et al., 2007)
	Phosvitin (ion-binding capacity, nutraceutical applications, antibacterial and emulsifying properties, antioxidant activity in medical and food applications)	(Chay Pak Ting et al., 2011; Jiang and Mine, 2000; Jiang et al., 2001; Vijeeta et al., 2004)
Lecithin and lecithin fractions	Purified phospholipid (PL) fractions (Food, cosmetic, medical/pharmaceutical.)	(De Ferra et al., 1997)
	Lysophospholipids, glycerophosphorylcholine, PLs with different acyl groups (Food, medical/pharmaceutical, laboratory/diagnostic.	(Kim et al., 2001; Pearce et al., 2002; Vijeeta et al., 2004)

2.1.3. Improving nutritional properties of egg yolk

Hen eggs have been used as food by human beings all over the world in such a variety of ways. Eggs obtained by conventional production represent important contributions to our diet. Indeed, the findings of a recent survey in the USA (Song and Kerver, 2000) showed that eggs contributed to almost 10% of the daily intake of energy and vitamin B6, 10-20% of folate and total saturated and polyunsaturated fat, 20-30% of the vitamins A, E and B12. However, recent investigations showed that it is possible to increase the amounts of certain nutrients in eggs and making them more like "free range" eggs (Bourre and Galea, 2006) or even further increasing these concentrations to produce "designer" eggs (Surai et al., 2000). The term designer eggs were assigned to those in which the content has been modified from the standard eggs as a new type of functional food (Surai and Sparks, 2001). Designer eggs represent over 5% of all shell eggs sold in Canada and United States (Leeson and Caston, 2003).

Consequently, modification or enrichment of eggs can be considered as an innovative approach in egg industry since improvement of egg composition by dietary means represent excellent vehicles for incorporation of several health promoting components in human diet.

2.1.3.1. Altering fatty acid content of egg

The total lipid of the egg yolk cannot be changed by manipulating hens' feed formulation. However the composition of fatty acids can be altered. Indeed, eggs are consumed all over the world and studies have shown that the fatty acid profile of egg is fairly easy to manipulate (Cachaldora et al., 2008; Lewis et al., 2000; Surai and Sparks, 2001). Commercial table eggs contains high amount of omega-6 polyunsaturated fatty acids (PUFA) but they are poor source of omega-3 fatty acids. Consequently, the production of omega-3 enriched eggs was practiced in two ways. The first consists to increase the level of linolenic acid in egg which is the precursor of docosahexaenoic acid (DHA). To achieve this goal, the hen's diet is usually fortified with flaxseeds or linseeds and subsequently the resulted eggs will be enriched with alpha-linolenic acid (ALA) (Ferrier et al., 1995; Van

Elswyk, 2007). The other alternative approach in enhancing the level of omega-3 levels in egg was done by including pre-formed DHA in hen's diet usually in the form of fish oil (Leskanich and Noble, 1997).

The content of omega-3 low chain PUFA in eggs has been successfully increased by offering fish oil to laying hens, but its addition to the diet at levels above 1.5% affects the sensory quality of eggs since "fishy" off-flavor were reported (Van Elswyk, 2007; Woods and Fearon, 2009). Moreover, flaxseed has been a popular source of omega-3 PUFA for animal feeds but there have been reports that eggs from hens offered flaxseed also have a fishy odor or taste, similar to that found in eggs from hens fed with fish oil (Woods and Fearon, 2009). Moreover, an increase in the content of omega-3 PUFA in the egg may increase its susceptibility to lipid oxidation, and although this is not a problematic issue in shell eggs (Marshall et al., 1994), it is a problem which arises in the processing of eggs by spray drying for use in the food industry.

Consequently, incorporation of new feeds in hen died is necessary to improve egg composition while limiting organoleptic disadvantages. In this context, novel feeds such as naked oats, camelina, hemp, chia and the daisy plant are investigated in order to increase levels of beneficial (unsaturated fatty acids) UFA in eggs (Woods and Fearon, 2009). Including these fatty acid sources in the diet of hen improved the fatty acid profile of eggs by increasing the ratio of UFA:SFA and decreasing the ratio of n-6:n-3 fatty acids.

2.1.3.2. Cholesterol-reduced eggs

Consumption of eggs has been reduced by the public as eggs are a rich source of dietary cholesterol and experimental evidence showed that dietary cholesterol increased serum cholesterol which is associated with the risk of coronary heart disease (Weggemans et al., 2001). The cholesterol content of the yolk is difficult to manipulate. However, different strategies have been practiced in order to produce low-cholesterol eggs. Some studies aimed in reducing the amount of cholesterol per egg by decreasing the size of the yolk or altering the lipid profile of the yolk. Cobos et al. (1995a) found no significant difference in cholesterol content of egg yolk obtained from two distinct strains of laying hens fed with

four diets containing different fatty acid profiles. Consequently, these results suggested that the fatty acid composition of the diet does not affect the cholesterol level in yolk. Sarker et al. (2005) conducted a study to evaluate the effect of dietary tamarind on serum and egg yolk cholesterol concentration modifications and overall performance in different layer strains. The result of their attempts showed that, although serum cholesterol concentrations decreased quadratically with increasing dietary levels of tamarind, the concentrations of cholesterol in the yolk were unaffected by these treatments (Sarker et al., 2005).

2.1.3.3. Vitamin- and mineral-enriched eggs

The cardio protective benefits of vitamin E encouraged researchers to investigate the possibility of enriching eggs with vitamin E through a targeted manipulation of hen diets (Meluzzi et al., 2000). Addition of vitamin E in the hen's diet at 200 mg/kg of feed has been found to have a positive effect on oxidative stability of shell eggs storage (Galobart et al., 2001). A substantial increase in the amount of vitamin E in the yolk was achieved linearly as dietary supplemented vitamin-E increased in hen's diet (Meluzzi et al., 2000).

Vitamin D plays an important role in the formation and maintenance of bone. Vitamin D is a generic term that refers to cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2). The two main sources that supply vitamin D are the action of sunlight on the skin and through the diet. In many cases the dietary fortification is requirement in people with restricted exposure to ultraviolet light. Eggs are among the limited number of natural foods that contain both vitamin D. Different studies shows that the cholecalciferol content of eggs is proportional to the level of added cholecalciferol in hen feed. The studies on the vitamin D deposition pattern in eggs showed that 8 to 13 days of high dietary cholecalciferol supplementation (11,200 and 12,000 IU/kg feed) is enough to reach the top cholecalciferol content in egg (30 μg/100 g egg yolk) (Mattila et al., 2004). Besides, it was observed that high doses of vitamin D (>15,000 IU/kg feed) were not toxic for hens and did not affect the sensory or functional properties, fatty acid composition, or the egg quality parameters such as egg shell strength (Mattila et al., 2004).

Carotenoids are known to provide a range of health benefits. Studies were conducted to observe the effectiveness of supplementation of hen's diet with a carotenoid-rich extract of alfalfa on egg composition. The result showed that eggs contained up to 22 times more carotenoids than the non-enriched eggs (Karadas et al., 2005).

Selenium is incorporated into selenoproteins that have a wide range of pleiotropic effects, ranging from antioxidant and anti-inflammatory effects to the production of active thyroid hormone. Selenium (Se) deficiency is recognized as a global problem which urgently needs resolution. In particular, developments and commercialization of organic forms of selenium have initiated a new era in the availability of selenium-enriched products. It has been shown that egg selenium content can easily be manipulated to give increased levels, especially when organic selenium is included in hen's diet at levels that provide 0.3-0.5 mg/kg selenium in the feed (Fisinin et al., 2009). As a result, the technology for the production of eggs delivering ~50% (30-35μg) of selenium recommended daily allowance (RDA) has been developed and successfully tested (Yaroshenko et al., 2003).

In order to solve the iodine deficiency problem, several studies were conducted to investigate the possible use of laying hen feed enriched with iodine for the production of iodine-enriched eggs. Iodine was included in feed formulation as KIO_3 or in the form of seaweed (Kaufmann et al., 1998). The findings showed significantly increased in egg iodine concentration depending on iodine intake after a 2 week period. Although the bioavailability of iodine from seaweed is less compared to KIO3 (50-60%), feeding seaweed increased iodine concentrations in egg yolk and egg white significantly. The mean feed conversion rate was comparable (feed intake per produced egg mass) independent of iodine supplementation (KIO3 or seaweed) (Kaufmann et al., 1998). Iodine-enriched eggs were also produced by chickens fed a diet containing kelp (Garber et al., 1993). These eggs contained an average of 711 μg iodine/egg and they were effective in reducing plasma cholesterol in humans and laboratory animals.

Other attempts were also done to investigate the efficiency of transfer of dietary iron sources to eggs of laying hens (Park et al., 2004). Average iron enrichment of 18% was achieved after feeding hens with iron fortified feed for 15 days.

2.1.3.4. Folate-enriched eggs

Following the growing concern about the possible health risks of high doses of synthetic folic acid attempts has been directed to other strategies in order to increase the consumption of natural folates (Ulrich and Potter, 2006). One of the effective solutions in enhancing natural folate in food was the enrichment of folate in eggs (Dickson et al., 2010; Hebert et al., 2005; House et al., 2002; Sherwood et al., 1993). The finding of the pre-mentioned studies have shown that the folate content of eggs can be increased significantly through supplementation of the laying hen diet with synthetic crystalline folic acid (FA). By feeding hens for at least 3 weeks through supplemented diet with 4 mg FA/kg, the level of folate concentration in the egg can be increased by about 2 to 2.5 fold compared to birds fed the basal diet (Dickson et al., 2010; Hebert et al., 2005; House et al., 2002). The majority of the supplemented FA was converted to the natural form of folate (5-methyltetrahydrofolate or 5-MTHF). Because 5-MTHF is the biologically active form of folate, the enzymatic reduction and methylation processes required for the metabolic utilization of FA are not pre-requisites for its consumption. The Food and Nutrition Board (1998) places an upper limit on synthetic FA in fortified foods conversely the 5-MTHF is considered with no health risk. The introduction of folate-enriched eggs can improve the population's intake of natural folates without having the same safety concerns associated with synthetic FA. In previous studies, FA addition to hen's diet increased egg folate concentrations and resulted into an enriched egg containing 50-60 μg dietary folate which is equivalents or approximately 12.5-15% of the recommended dietary allowance for adults (Food and Nutrition Board, 1998). The relative bioavailability of folate in eggs is considered to be higher than or equal to 100% compared to FA (House et al., 2003a). The supplementation of 5-MTHF was compared to FA in order to characterize the biochemical changes in egg folate concentration and indices of folate status (Tactacan, 2011a). Experiments performed by Tactacan (2011) demonstrated that FA and 5-MTHF feed supplementation have equivalent effects in enhancing egg folate concentrations, improving folate status, and the overall activity of the different folate-dependent enzymes.

Attempts to further increase the level of folate in eggs have proven unsuccessful since folate levels reach a maximum plateau due to the presence of saturable processes during intestinal folate absorption (Said, 2004; Said et al., 2000).

2.2. FOLATE AND FOLIC ACID

2.2.1. Historical perspective

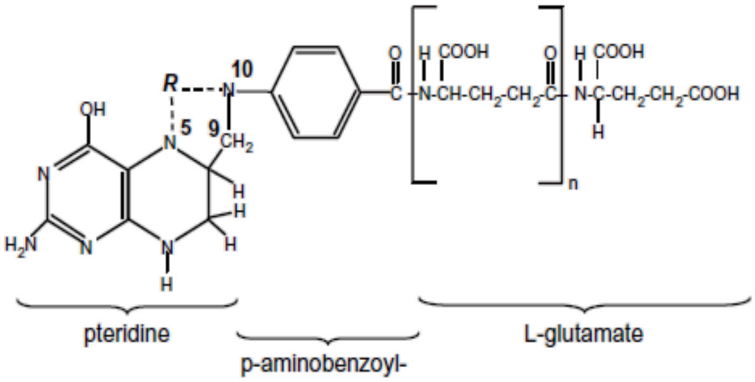
Folate, discovered in 1931, is a water-soluble B vitamin which is called vitamin B9. Folic acid received its name in 1941 when it was isolated from spinach (Table 2.3) and its structure was determined in the mid-1940s. The compound was consequently synthesized in pure crystalline form in 1943 and this finding proved that folic acid was composed of a pteridine ring, paraminobenzoic acid and glutamic acid. Afterward, it became evident that natural folates usually differed from pteroylglutamic acid. Today, folic acid refers to the fully oxidized chemical compound which does not exist in natural foods. The term ‘folate’ is designated to the large group of compounds having the same vitamin activity and includes natural folates and folic acid (Hoffbrand and Weir, 2001).

Table 2.3. The chronology of folic acid discovery-Adapted from Hoffbrand and Weir (2001)

1930	Wills & Mehta	Yeast extract prevented the dietary anaemia in rats
1931	Wills	Yeast or Marmite prevents macrocytic anaemia of pregnancy
1932	Vaughan & Turnbull	Marmite corrects anaemia of coeliac disease
1938	Wills & Evans	Purified liver extracts do not correct nutritional, pregnancy or macrocytic anaemia
1938	Day et al.	Vitamin M corrects nutritional anaemia in monkeys
1940	Snell and Peterson	Norit eluate factor-factor absorbed from yeast or liver is growth factor for *Lactobacillus casei*
1941	Mitchell et al.	The term Folic acid coined and shown to be a growth factor for *Streptococcus lactis R* (*S. faecalis*)
1943	Fullerton; Watson & Castle	Idiopathic steatorrhoea megalobastic anaemia responds to crude liver extracts or yeast extract
1943	Wright & Welch	Enzyme hydrolysing folate polyglutamates to monoglutamates-folate conjugase
1944	Binkley et al.	Yeast extracts effective as a source of vitamin BC only 2–5% being active for *L. casei*. Required enzymatic digestion to balance activity
1945	Angier et al.	Synthesis of folic acid and using the term pteroylglutamic acid
1945	Day et al.	Purified *L.casei* factor is vitamin M
1946	Pfiffner et al.	Naturally occurring folate in liver is a heptaglutamate

2.2.2. Structure and biochemistry

Folate is the generic term for folic acid (pteroylmonoglutamic acid) and related compounds exhibiting the biological activity of folic acid. The terms folacin, folic acids, and folates are used only as general terms for this group of heterocyclic compounds based on the N-[(6-pteridinyl)methyl]-p-aminobenzoic acid skeleton conjugated with one or more L-glutamic acid residues. Folates can consist of a mono- or polyglutamyl conjugate. The folates from most natural sources usually have a single carbon unit at N-5 and/or N-10; these forms participate in the metabolism of the single-carbon pool. The compound called folic acid is not present in living cells, being rather an artificial and manmade form of the vitamin. Folic acid (pteroylmonoglutamic acid) is an orange-yellow crystalline substance that is soluble in water but insoluble in ethanol or less polar organic solvents. It is unstable to light, acidic or alkaline conditions, reducing agents, and to heat (except in dry form). The UV absorption spectra of the folates are characterized by the independent contributions of the pterin and 4-aminobenzoyl moieties; most have absorption maxima in the region of 280–300 nm (Lavoisier, 2008).



Name	Abbreviation	Substituent	Position
5-methyltetrahydrofolate	5-CH_3-H_4 folate	-CH_3	N-5
5-formyltetrahydrofolate	5-HCO-H_4 folate	-HCO	N-5
10-formyltetrahydrofolate	10-HCO-H_4 folate	-HCO	N-10
5-formiminotetrahydrofolate	5-CHNH-H_4 folate	-CH=NH	N-5
5,10-methylenetetrahydrofolate	5, 10-CH_2-H_4 folate	-CH_2-	N-5 & N-10
5,10-methenyltetrahydrofolate	5, 10-CH^+-H_4 folate	-CH=	N-5 & N-10

Figure 2.4. Structure of native food folates and their substituent groups and positions. n:number of glutamates. Adapted from Witthöft (2011)

Naturally occurring folates, such as those found in foods and body tissues, exist in different forms. They contain a fully-reduced pteridine ring together with additional glutamic acid molecules (Figure 2.4). They are usually substituted by different one-carbon units at the N-5 or N-10 positions, or have a single-carbon bridge spanning these positions. These one-carbon units can be at the oxidation level of methanol (5-methyltetrahydrofolate), or formaldehyde (5, 10-methylenetetrahydrofolate), or formate (5, 10-methenyltetrahydrofolate) (Scott and Weir, 1993).

The 5-methyltetrahydrofolate (5-MTHF) is the predominant natural form of folate in fruits and vegetables (Vahteristo et al., 1997). In animal products the 5-MTHF and tetrahydrofolate are predominate (Vahteristo et al., 1997) while cereal products contain 5-MTHF, 5-formyltrahydrofolate, and 10-formlytrahydro folate (Pfeiffer et al., 1997).

2.2.3. Importance of folate in human diet

The naturally occurring form of folate lacks stability during food storage and preparation and thereby the stable folic acid (Eitenmiller et al., 2007) was used for supplements and food fortification. Folate has a crucial role as a one-carbon source for DNA, RNA synthesis and protein methylation (Stover, 2009). A number of genetic polymorphisms affect critical components of folate pathways and metabolism, and have been associated with an increased risk for neural tube defects (NTD) (Molloy et al., 2009). However, the exact mechanism by which folic acid reduces the risk of NTDs is not known and remains an active area of research (Crider et al., 2011a). NTDs occur when the neural tube fails to close early in embryonic development, resulting in damage to the exposed underlying neural tissue. These birth defects can result in significant morbidity and mortality depending on the location and severity of the lesion (Crider et al., 2011a). The most severe lesions observed with spina bifida cause a range of morbidities, including urinary and fecal incontinence and paralysis of the lower limbs (Sutton et al., 2008).

The relationship between NTD occurrence and folate deficiency encouraged scientist to demonstrate the link between folic acid intake and the risk of birth defects. A randomized control trial study found that the risk of NTD occurrence can be reduced by 70% when

women consume 400 µg of folic acid daily (MRC, 1991). In 1991, the Centers for Disease Control and Prevention recommended that women with a history of NTD affected pregnancy should consume 400 µg of folic acid daily before conception (CDC, 1991). Subsequently, in 1992, the U.S. Public Health Service recommended that all women of childbearing age consume 400 µg of folic acid daily through fortification, supplementation, and diet to prevent NTDs (CDC, 1991). In 1998, the Institute of Medicine (IOM) recommended that women at the age of becoming pregnant should consume additional 400 µg of folic acid daily from fortified foods or supplements to that obtained through a normal diet (1998). In order to increase the folic acid level in the diet of all women of childbearing age, the regulations for mandatory fortification of wheat flour with folic acid has begun. The mandatory folic acid fortification of enriched cereal grain products was authorized in 1996 in U.S. and fully implemented in 1998 (FDA, 1996). This program aimed to add 140 µg of folic acid/100 g of enriched cereal grain product in order to provide nearly 100–200 µg of folic acid/day for women of childbearing age (Quinlivan and Gregory, 2007).

Studies showed a significant decrease of 19%–32% in the prevalence of NTDs and spina bifida since the implementation of folic acid fortification in 1998 (Boulet et al., 2008). However, there are concerns about potential adverse consequences e.g. masking of B12 deficiency anemia, cancer and epigenetic changes, and circulating unmetabolized folic acid in the blood (Crider et al., 2011b).

Folic acid normally is reduced to tetrahydrofolate following uptake by the liver. If the folic acid is exceeded from tolerable upper intake level (1000 µg/day) (IOM, 1998), the non-metabolized folic acid will be found circulating in the blood. Synthetic folic acid is absorbed by passive diffusion and it should be reduced and then metabolized to 5-MTHF in the human mucosal cell and/or liver. Because the ability of conversion is limited, unmetabolized folic acid can appear in the systemic circulation, even after low-dose application (Pfeiffer et al., 1997). The debate addresses two main risk of long term consumption of high folic acid intake which could promote the formation of colorectal tumors (Kim, 2004) and masking the appearance of vitamin B12 deficiency anemia (Morris et al., 2010).

Considering the recently mentioned adverse effects of folic acid fortification, attention has been directed toward other solutions that might help increase population intake of natural folates, which do not carry with them the same safety concerns as synthetic folic acid. Thereby, scientists have developed other strategies to increase natural folate intakes in population without adverse effect of synthetic folic acid. Consequently, different works have been done to develop novel fortified foods in natural folates (Buttriss, 2005). Studies revealed that folate presents in fortified eggs is present in its natural form, mainly as 5-MTHF.Consequently, this strategy represents an efficient solution to increase folate intake of population, especially where long-term consumption of folic acid-fortified foods has been criticized. Among all vitamers of folate, 5-MTHF in the monoglutamate form has been reported by several researches as the only predominant form of folate in egg yolk (McKillop et al., 2003; Seyoum and Selhub, 1993). Synthetic crystalline folic acid supplemented into laying hen feed is deposited in the egg yolk (Sherwood et al., 1993).

Based on LC/MS analysis conducted by Strandler (2012) , the folate forms found in egg yolk were 5-MTHF (292µg/100g) whereas no folates were found in egg white (Strandler, 2012). This researcher stated that no polyglutamate forms were present in egg yolk. There is now interest for the use of 5-MTHF which cannot possibly mask the Vitamin B12 deficiency as an alternative to folic acid (Wright et al., 2001). Furthermore, 5-MTHF is the most abundant form of folate which has exceptional importance among all forms of folate for having dual function. House et al. (2003) also reported the relative bioavailability of folate in eggs is higher or equal to 100% compared to folic acid. Compared to green leafy vegetables, folates in animal products including eggs are also usually more stable during thermal treatment (McKillop et al., 2003). However, a significant obstacle in egg consuming is the perception that egg consumption is associated with a rise in blood cholesterol levels (Yaffee et al., 1991). Today, the need for new and biologically active ingredients for the development of novel functional foods is increasing. Egg yolk is a good source of biologically active folate in the form of 5-MTHF that can be separated while maintaining the activity of the molecule. Therefore, some experiments were performed to enrich 5-MTHF concentration in the egg that naturally contains 34 µg/egg. Indeed, it has been recently confirmed that it is possible to enhance the folic acid content of eggs by 2 to 4 fold by feeding hens a folic acid supplemented diet. A linear increase in egg folacin

content was described when crystalline folic acid was added from 0 to 2 mg/kg to hen diet. Then, egg folate levels reached a plateau at a concentration of approximately 45–50 μg/egg, with no additional increases until dietary concentrations reached 32 mg/kg. It has been also demonstrated that the folate in enriched eggs is stable during storage at 4 °C for 4 weeks (House et al., 2002). However, the amount of 45-50 μg/egg is still lower than the amount of recommended daily requirement of 400 μg per day which represent the consumption of ten eggs per day. Since it is not possible to reach this threshold, it becomes necessary to use fractionation processes to separate the egg yolk in order to extract and recover folate enriched fractions.

2.3. FRACTIONATION METHODS FOR DEVELOPMENT OF FOOD-DERIVED BIOACTIVES

Separation, extraction and concentration processes, used in the food, nutraceutical, pharmaceutical and health ingredient industries, are essential to recover bioactive components from their natural matrices. The separations techniques usually intend to achieve removal of specific components (e.g. residue and/or the extracted components) in order to increase the added value of the products. Separation techniques, such as filtration, evaporation, dehydration, solid-liquid and gas-liquid extraction processes, are based on the nature (liquid, solid or gaseous) and different physicochemical properties (density, solubility, electrostatic charge, particle or molecular size and shape) of the sample and target components. However, most of the processes involved are of a physical nature (Grandison and Lewis, 1996).

The separation processes may be batch or continuous. A single separation process involves the contact of the solvent with the food. The continuous processes may be single or multiple stage processes (Grandison and Lewis, 1996).

Separation techniques for solid foods can be classified into two categories. The first category includes solid-solid separation processes that require the separation or segregation of particles. The other processes involve removing the discrete solid particles from liquid, gas or vapors. In solid-solid separation, the fractionation technique can be achieved on the

basis of particle size from sorting of large food units down to the molecular level. The shape, electrostatic charge and degree of hydration are major factors that may affect the solid-solid separation (Brennan, 1990). The separation of liquid (oil, water, etc.) or air from solid matrix can achieve by processes such as pressing, extraction, dehydration, blanching.

The separation techniques from liquids are based on the important physical property of the fluid which is the viscosity of the fluid. The fluid is a substance which flows when it is subjected to a shear stress and the viscosity of the fluid is the ratio of the shear stress to shear rate (Lewis et al., 2000). Conventional filtration systems are being used in solid-liquid separations. The application of pressure filtration, vacuum filtration and centrifugal filtration can be applied in order to separate large amount of insoluble solids from a liquid matrix. Filtration techniques with the introduction of membranes with different pore size and molecular weight cut-off have become popular in food and biotechnology industries. The ultrafiltration membranes permit the passage of water and components of low molecular weight in a fluid but rejects macromolecules such as proteins. Solids also may separate from the liquids on the basis of particle size and density using settlement or using centrifugation (Brennan, 1990).

In the case of having immiscible liquids, such as egg yolk, the centrifugation in cylindrical bowls are the simplest methods to separate liquids of different densities. The tubular bowl or disc-bowl type centrifuges are usually used for liquid-liquid separation.

2.3.1. Overview of the separation methods in egg processing

The eggs, one of the few foods that are used throughout the world, are main ingredient of many food products owing to their multi-functionalities. Moreover, both egg white (albumen) and egg yolk are rich sources of biologically active components but only a few egg compounds (i.e. egg yolk antibodies and IgY, egg yolk phospholipids, lutein and zeaxanthin, cholin, lecithin, egg membranes and egg shell) have been successfully used in the formulation of food or non-food products. The food industry is the major user of egg products and the development of the egg product industry started in the early 1900s. The first product was frozen whole eggs, followed by separated whites and frozen yolks. By the

improvement of the equipment, technology and customer acceptance the industry progressed slowly until 1940 (Sim and Nakai, 1994).

The development of egg-breaking and separating machines was a major improvement in the efficient production of liquid-egg products. Each separator cup has a separate cracking assembly. The shells are transferred from breakers directly into a trailer or truck in another room and sometimes these shells are centrifuged to recover any adhering egg white. Egg products for freezing should be frozen or reduced to a temperature of -12ºC or lower within 60 h from time of breaking or pasteurization. Five main egg products that are producing by egg-breaking plants are liquid egg white, liquid egg yolk, liquid whole egg, egg shell and inedible egg products (Cotterill and McBee, 1995).

The egg white makes up of 66% of the liquid weight of the egg and it contains 88-90% water. Proteins are major component of albumen solids (10-11% of white weight) and the carbohydrates mainly glucose (0.8-1%), minerals (0.5%) and lipids (0.03%) are minor components. The glucose is the main free sugar in egg white and it should be usually removed through enzymatic hydrolysis or bacterial fermentation before drying of egg white. The egg white contains high water content and it should be concentrated before spray drying in order to reduce the energy cost. Membrane processes, especially reverse osmosis and ultrafiltration with low molecular weight cut-off, were investigated in order to concentrate egg before drying. These processes have been commercially applied to the concentration of egg white (Bergquist, 1995).

Egg white proteins have significant importance due to their high nutritional, functional and biological properties. Several processes of protein isolation have already been suggested but very few methods have been developed on an industrial scale (Nau et al., 2010). Lysozyme, ovotransferrin and avidin are the three proteins commercially separated from egg white. Lysozyme from hen egg is small protein with molecular size of 14 kDa with strong basic characteristic. The commercial procedures of lysozyme isolation are based on the very high isoelectric point of lysozyme and consist of the selective precipitation of lysozyme and extraction using ion-exchange chromatography. The selective precipitation of lysozyme combines the adjustment of the pH to 10 (close to isoelectric point of protein) and the addition of 5% sodium chloride to increase ionic strength and favoring

precipitation. The ion exchange chromatography mainly cation-exchange chromatography has been used on an industrial scale to isolate lysozyme (Gésan-Guiziou and Rizvi, 2010).

Ovotransferin from egg white has been separated based on liquid chromatography. This protein has a neutral isoeletric point (pI 6.2-6.7) and its extraction can be performed either by cation- or anion-exchange chromatography (Guerin-Dubiard et al., 2010).

Avidin is important protein in egg white which has strong affinity to bind biotin (vitamin H or B8). Several separation techniques based on chromatographic separations have been studied based on ion exchange or affinity properties (Durance et al., 1990).

Even though many of the egg compounds have been studied only a few of them (eg., IgY, lysozyme, yolk phospholipids) are utilized in food, and the pharmaceutical industries.

The immunoglobulin (IgY) of egg yolk, Gamma-livetin (egg antibody) and phospholipids (phosphatidylcholine, phosphatidylethanolamine) are commercially extracted from egg yolk. The isolation of Gamma-livetin have been achieved by precipitation with polyethyleneglycol and the uses of ion-exchange chromatography (Schade et al., 2005). Gamma-livetin has been developed on an industrial scale for more than twenty years. The application of this egg antibody is promising as functional tool for use in immunoassays in order to quantify toxins or pathogenic viruses. Various extraction methods were reviewed by Schade et al. (2005) in details. Two isolation methods have been developed. The first method comprises the precipitation of the Gamma-livetin by polyethyleneglycol and the second one uses ion-exchange chromatography leading to 70% recovery with 60% purity. In both cases, the protein is first recovered in the soluble phase of the yolk by water dilution (6 times; pH 5, 4ºC for 6 h).

Different extraction methods for egg yolk lipids have been presented. Most of them are based on the use of organic solvents. However, those methods are not compatible tor ultimate use in food or pharmaceutical applications due to possible solvent residues in final products.

The egg yolk phospholipids have several industrial applications in food, nutritional, pharmaceutical, and cosmetic fields. Industrial extraction and separation of phospholipids

from egg yolk is mainly based on the use of organic solvents and hydrocarbons whereas these molecules are typically insoluble in acetone. Moreover, the latter characteristic allows the separation of the accompanying lipids in order to increase the purity of the phospholipids. Thus, ethanol is also used to increase phosphatidylcholine (PC) and phosphatidylethanolamines (PE) since these molecules have affinity for this organic solvent. Thereby, a method for large-scale preparation of phospholipids from fresh egg yolk was developed by Juneja et al. (1994) by addition of combined solvent (acetone and ethanol) followed by filter press separation. By using this process, the final phospholipid fraction had similar composition as phospholipids in egg yolk (80-85% PC; 10-15% PE). However, due to solvent toxicity used in extraction process and the problematic of waste storage, the application of alternative and eco-friendly techniques such as supercritical carbon dioxide fluid was introduced.

2.3.1.1. Supercritical Fluids – A sustainable Separation Technology

When a substance is compressed and heated above its critical pressure and temperature, it has liquid-like density and gas-like viscosity. When both of these parameters exceed the critical values, the substance in this state is called a supercritical fluid (SCF). Carbon dioxide (CO_2) is the most common medium in supercritical state for the extraction of natural compounds. The critical constants for supercritical carbon dioxide (SC-CO_2) are 73.8 bar and 31.1°C. The most common and recognized SCF application is the supercritical fluid extraction (SFE). The SC-CO_2 provides a low-cost, non-toxic, eco-friendly and well-penetrating medium for processing foodstuffs, nutraceuticals, and pharmaceuticals (Aro, 2007).

The proteins of egg albumen are almost insoluble in SC-CO_2 and therefore not extractable. During the last two decades many SCF applications related to modification of different lipid compounds in egg yolk have been reported. Most of these applications are based on consumer demand for healthier food products. Partial delipidation or decholesterolification of egg yolk powder increases its nutritional value if the texture and the sensory properties of final product remain unaffected during the process (Aro, 2007). The application of SC-

CO_2 has been already used in extraction of cholesterol and other lipids from dried egg yolk (Froning et al., 1990). Based on several studies, both nonpolar and polar lipids can be processed using supercritical fluids if ethanol as carrier is added to the fluid. In several studies, mixtures of phospholipids with various co-extractives solubilized in ethanol are obtained through SC-CO_2 separation processes (Elst et al., 2003; Shah et al., 2004).

A method has been described at pilot scale for the production of pure phospholipids from egg yolk using a technique similar to the supercritical anti-solvent technique (Aro et al., 2009). In this pilot scale process, commercial egg yolk powder was used as starting material.

2.3.1.2. Fractionation of egg yolk proteins and peptides

The application of egg protein fractionation processes is becoming more interesting because of their technological and functional properties. There is a need to develop efficient, simple and cost-effective methodologies for isolation and purification of egg proteins and peptides. Several available technological choices for fractionation of egg proteins and their peptide are listed in Table 2.4.

Table 2.4. Technological approaches and processes to fractionate peptides and proteins from egg.

precipitation	Isoelectric precipitation
	Salting-out
	Organic solvents
Chromatography	Gel permeation
	Ion-exchange
	Reverse-phase/hydrophobic
Membrane separations	Microfiltration
	Ultrafiltration
	Nanofiltration

Adapted from: (Chay Pak Ting et al., 2010)

The present industrial processes comprise three main approaches of precipitation, chromatography and membrane process alone or in combination. Consequently, the physiochemical characteristics of the protein/peptides must be known in order to select the appropriate technology and parameters.

I. Precipitation by ionic strength or pH modification

Purification of the immunoglobulin Y (IgY) involves removal of lipid and lipoproteins from egg yolk. Various strategies involving detergents such as SDS and polysaccharides, solvents and polyethylene glycol were used to remove lipids from egg yolk. Salt precipitation can thereafter be achieved for IgY purification. Ammonium sulfate and sodium sulfate were used and concentration levels were dependent on the IgY yield and purity. The precipitation by salt has drawbacks of having a by-product with high salt concentration. Besides, several solubilization and crystallization cycles must be completed to have highly purified final protein product (Chay Pak Ting et al, 2010).

II. Precipitation by organic solvent

Precipitation of proteins by organic solvents results in important modification of the dielectric constant of the medium and to the weakening of protein interactions with water. Organic solvents have affinity for the hydrophobic surfaces of the proteins and this result in denaturation of the proteins along with the precipitation. However, low temperatures (-5 to 0ºC) are necessary to minimize protein denaturation. In a large-scale sequential separation method, the egg yolk were fractionated into the IgY, phospholipids and neutral lipids by using water, ethanol, and hexane. The water soluble and water non-soluble fractions of yolk were separated in first step through centrifugation and in the next step the IgY were concentrated and separated by ultrafiltration (Ahn et al., 2006).

III. Gel-permeation and Ion exchange chromatography

Gel-permeation chromatography (GPC) separates the proteins based on the differences in their size. GPC was usually used in combination with other chromatographic procedures. GPC has been used in isolation of phosvitin from delipidated egg yolk proteins (Tsutsui and Obara, 1984). Separation of different sizes of proteins even with molecular sizes close

threshold is possible with GPC. However, the large-scale of GPC processes may not be easily applicable because of the fragile nature of many soft gels used in GPC. Besides, the interaction of gels with proteins reduces the potential utilization of GPC for egg protein fractionation (Chay Pak Ting et al., 2010).

Ion exchange chromatography (IEC) is the most widely used large-scale method for the purification of proteins and other charged molecules. Phosvitin purification method was described by Castellani et al. (2003), was based on two steps extraction method. The first step was based on the insolubility of Mg^{2+}/phosvitin salts and the second step was based on ion-exchange chromatographic fractionation without using organic solvents. The purification procedures resulted in highly purified (>98%) and metal-free α- and β-phosvitin.

Consequently, the IEC method is suitable for fractionation of egg proteins with high purity but the application of IEC on industrial-scale are only concentrated on major egg white proteins and not the egg yolk proteins (Chay Pak Ting et al., 2010).

IV. Membrane processes

Membrane based separation techniques are powerful tools for fractionation and purification of proteins and bioactive peptides. Compare to chromatographic methods, membrane separation techniques are less expensive and are easily adapted to an industrial scale. The surface properties of the membrane are main factors for the successful protein fractionation besides the physicochemical parameters such as pH and salt concentration (Kim and Nakai, 1996). Ultrafiltration (UF) membranes can be used as preliminary step for the removal of enzymes and non-hydrolyzed proteins, and to further fractionate the peptide mixture. Various combinations of precipitation and UF using selective membranes can be applied for the fractionation of food proteins. The membranes have been successfully applied for peptide enrichment (Chay Pak Ting et al., 2010). The membranes with negatively charged materials such as nanofiltration has been already successfully used in order to desalt and fractionate acidic peptides from hydrolysate mixture (Chay Pak Ting et al., 2007).

A water-soluble plasma protein from egg yolk granules was obtained with various simple water dilutions followed by centrifugation or filtration. In IgY recovery two factors of pH and egg yolk dilution were critical. A serial filtration approach including dilutions, paper filtration and delipidation using hydrophobic filters or using different UF membranes was developed by Kim and Nakai (1998, 1996). An ultrafiltration-based approach was performed in order to prepare phosvitin from delipidated egg yolk proteins. By applying the diafiltration technique, the researchers attempted to desalt and concentrate phosvitin. The results indicated that desalting of phosvitin can be achieved at laboratory scale by using 10- and 30-kDa molecular weight cut-off (MWCO) polyethersulfone membrane with a few loss of protein in the permeate side (Chay Pak Ting et al., 2010).

Moreover, reverse osmosis and ultrafiltration were used as pre-concentration step for the subsequent separation of egg protein by ion-exchange technology.

There is a great interest in removing cholesterol from yolk, with strong marketing advantages for resulting product. But, despite its relatively low molecular weight, this has not been achieved by UF (Lewis, 1996).

2.3.1.3. Fractionation of egg yolk using centrifugation techniques

Based on research studies, individual components of yolk are difficult to separate and only plasma and granules can be easily fractionated from yolk at an industrial scale (Anton, 2007b; Anton et al., 2001). Over the last years, different studies concentrated on fractionation of egg yolk (Anton, 2013; Laca et al., 2011, 2010; Navidghasemizad et al., 2014; Strixner and Kulozik, 2013) and characterization of granules and plasma according to their composition and properties.

In many processes, the use of sedimentation to separate two liquids or a liquid and a solid does not progress rapidly enough to accomplish separation efficiently. In these types of applications, the separation can be accelerated through the use of centrifugal force. Centrifugation is a mechanical process that uses a spinning medium to separate one or more components of a sample according to density or size. One of the main approaches to the

development of the egg industry is fractionation of egg components and move forward with new innovative applications.

In native conditions, yolk is constituted of a continuous aqueous phase (plasma) and insoluble denser structures with a size ranging from 0.3 to 2 mm (granules) (Guilmineau and Kulozik, 2006). Plasma is composed of LDLs and livetins, whereas granules are mainly constituted by HDLs, phosvitin and LDLs (Anton, 2013). Due to the differences in size and density, the egg yolk main fractions granula and plasma can be separated by centrifugation according to the method described for the first time by Mc Bee and Cotterill (1979). In their protocol, the egg yolk was first diluted (1:2, w/w) in an isotonic sodium chloride solution (0.17 M NaCl) and stirred gently for 1 h before centrifugation at 10.000 g for 45 min at 10°C. The supernatant plasma was collected, and the sedimented granules were washed by re-suspending them in twice their volume in 0.17 M NaCl solution. To increase the purity of plasma and washed granule fraction, another step of centrifugation was performed by using the pre-mentioned conditions.

Later, different fractionation methods have been developed in order to broaden the applications of egg yolk. However, many of those methods are based on the use of organic solvents (Kwan et al., 1991; Sheumack and Burley, 1988). In most applications, fractionation methods based on the use of organic solvents are not completely compatible for ultimate use as food or pharmaceutical ingredients due to the possible solvent residuals in the final products. Other approaches to isolate the egg yolk compounds were based on heating methods (Liot, 2002). Nevertheless, protein denaturation caused by heat treatments represents a major problem according to the final products recovered.

The fractionation procedure of egg yolk into granule and plasma is always based on discontinuous fractionation methods by using two basic steps: dilution and centrifugation. Different studies have been employed the pre-mentioned procedure with some modifications in yolk dilution factor and/or centrifugation conditions (Anton et al., 2003, 2000a; Castellani et al., 2003; Freschi et al., 2011; Kiosseoglou and Paraskevopoulou, 2005; Le Denmat et al., 1999; Sousa et al., 2007). Usually, phosphate buffer for egg yolk dilution instead of NaCl solution (Rojas et al., 2006) or DI H_2O were used (Kwan et al.,

1991; Laca et al., 2010; Lei and Wu, 2012; Merkle and Ball, 2001) for egg yolk dilution before centrifugation procedures.

Table 2.5 reviewed the fractionation methods, based on centrifugation technique, applied to separate the egg yolk granule from the plasma.

Table 2.5. Fractionation of egg yolk into granule and plasma based on centrifugation methods

Dilution ratio (Yolk:Solvent)	Solvent	Pretreatment	Centrifugation conditions	References
1:1	Salin solution (0.17 M NaCl)	-	4°C/10,000 g/15 min	(Mc Bee and Cotterill, 1979)
1:10	DI H_2O	Stored diluted yolk overnight (4°C)	4°C/10,000 g /15 min	(Kwan et al., 1991)
1:1	Salin solution (0.16 M NaCI)	Mixed diluted yolk (5min) and dialyzed against DI H_2O (4°C ;12h)	10°C/13,000 g /1 h	(Dyer-Hurdon et al., 1993)
1:4	Saline solution (0.16 M NaCl)	-	4°C/8000 g /30 min	(Aluko and Mine, 1998)
1:1	Saline solution (0.17 M NaCl)	Mixed diluted yolk with a magnetic stirrer (1h; 4°C)	4°C/10,000 g /45 min	(Anton et al., 2003; Castellani et al., 2003; Freschi et al., 2011; Le Denmat et al., 2000; Moussa et al., 2002; Sousa et al., 2007)
1:3	Saline solution (0.17 M NaCl)	Mixed diluted yolk with a magnetic stirrer (1h; 4°C)	4°C/10,000 g /45 min	(Kiosseoglou and Paraskevopoulou, 2005)
1:1	Phosphate buffer	Mixed diluted yolk with a magnetic stirrer (1h; 4°C)	4°C/10,000 g/45 min	(Rojas et al., 2006)
1:1.5	DI H_2O (adjust the pH to 7 with 1 N NaOH)	Stored diluted yolk overnight (4°C)	4°C/10,000 g /45 min	(Laca et al., 2010)
1:1	DI H_2O	Mixed diluted yolk with a magnetic stirrer (1h; 4°C)	4°C/10,000 g /45 min	(Lei and Wu, 2012)
1:2	0.15 M NaCl	Mixed diluted yolk with a magnetic stirrer (1h; 10°C)	10°C/10,000 g /45 min	(Strixner and Kulozik, 2013)
1:1	Saline solution (0.17 M NaCl)	Mixed diluted yolk with a magnetic stirrer (1 h;10°C)	10°C/10,000 g /45 min	(JIN et al., 2013; Le Denmat et al., 2000)
1:10	MilliQ water (adjust the pH to 6 with 1 M HCl)	Mixed diluted yolk with a magnetic stirrer (1h; 4°C)	4°C/10,000 g /15 min	(Navidghasemizad et al., 2014)

Other separation methods, such as ultracentrifugation, were applied for separating the low density lipoproteins (LDLs) from egg yolk. Independent of the pH and ionic conditions, LDLs are soluble in aqueous solution owing to their low density (0.982 g/cm^3) close to the water. However, ultracentrifugation is extremely time consuming (several days of centrifugation is needed), and does not allow high extraction yield (only few grams of LDL), and cannot be adapted to an industrial-scale (Anton, 2007c).

Different authors pointed out other factors which might affect the composition of granules and plasma separated through different fractionation techniques naming as: storage time of egg (Freschi et al., 2011), diet formulations of hen, hen's species or age of hen (Li-Chan and Kim, 2008).

The composition of plasma and granules separated based on centrifugation techniques are summarized in Table 2.6. The egg yolk fractionation process based on centrifugation techniques shows to be an appropriate method to obtain products with potential uses in some interesting applications (Laca et al., 2010). So far, the applications of egg yolk plasma has not been at industrial or lab scale as food ingredient. However, the properties of plasma being as gelling and emulsifier agent has been investigated (Anton and Gandemer, 1997; Anton et al., 2003; Nilsson et al., 2007, 2006; Sirvente et al., 2007).

Table 2.6. Summary of granule and plasma composition after fractionation by centrifugation (values are expressed as a percentage of dry basis)

Yolk dry matter (%)	Dry matter (%)	Protein	Lipid	Cholesterol	Yolk dry matter (%)	Dry matter (%)	Protein	Lipid	Cholesterol	References
Granule					Plasma					
25	-	59	33	-	-	-	-	-	-	(Causeret et al., 1991)
25	-	64	34	1.4	75	-	24	79	2.4	(Dyer-Hurdon et al., 1993)
19-25	-	-	-	0.5	-	-	-	-	-	(Le Denmat et al., 2000)
19-23	44	64	31	0.5	-	-	-	-	-	(Anton, 2007b)
-	-	-	-	-	77-81	-	25	73	-	(Huopalahti et al., 2007a)
-	-	60	34	-	78	51	18	80	-	(Li-Chan and Kim, 2008)
29	41	58	40	0.7	-	-	-	-	-	(Laca et al., 2010)
22	-	53-56	40-43	-	77-81	-	23	80	-	(Freschi et al., 2011)
-	57	50	22	-	77-81	-	25	73	-	(Navidghasemizad et al., 2014)
-	34-36	48-65	-	-						(Strixner and Kulozik, 2013)

In different studies the granules were characterized being low in cholesterol content comparing to native yolk and plasma (Anton, 2007a; Dyer-Hurdon and Nnanna, 1993; Jin et al., 2013; Laca et al., 2010). One of the main characteristic of granule fraction is the presence of phosvitin protein in its protein composition. Over the last years, in different studies phosvitin was separated from egg yolk and it was characterized base on its different functional and biological properties e.g. antioxidant, antibacterial, anti-inflammatory and anticancer activities (Castellani et al., 2003; Chay Pak Ting et al., 2011; Lei and Wu, 2012; Chay Pak Ting et al., 2010; Xu et al., 2007).

One of the major characteristic of granule and plasma that has been studies following the fractionation of egg was identification of protein profile of each fraction by SDS-PAGE or 2D gel electrophoresis analysis. More than 119 proteins have been identified in proteomics analysis of egg yolk (Mann and Mann, 2008). Many studies characterized the main apoproteins in granule and plasma faction by using SDS-PAGE analysis. Most of the authors have reported the presence of five major bands in the protein profile of granules corresponding to the apoproteins of HDL and phosvitin (Anton et al., 2000; Castellani et al., 2003b; Guilmineau et al., 2005; Freschi et al., 2011; Laca et al., 2010; Le Denmat et al., 2000; Lei and Wu, 2012; Mine, 1998; Orcajo et al., 2013; Strixner and Kulozik, 2013) while a total main nine bands were reported in the SDS-PAGE protein profile of plasma corresponding to LDL apoproteins and livetins (Anton et al., 2003, 2000; Guilmineau et al., 2005; Freschi et al., 2011; Huopalahti et al., 2007; Le Denmat et al., 2000; Mine, 1998; Strixner and Kulozik, 2013).

Differences were observed in the molecular sizes of protein bands in granule in the reports published by authors. List of protein bands detected in different studies and identified by using SDS-PAGE analysis are listed in Table 2.7. The 2D gel electrophoresis analysis of granule fraction was limited and more research is needed in order to characterize the protein profile of this fraction using this technique.

Table 2.7. Protein bands observed in the SDS-PAGE analysis of granule and plasma fractions

Granule		Plasma		
MW (KDa)	**Identification of protein bands**	**MW (KDa)**	**Identification of protein bands**	**Reference**
215	Lipovitellin	57	livetin	(Mine, 1998)
105	Lipovitellin	10-180	6 major polypeptides	
81	Lipovitellin	-	Several unidentified	
38-48	Phosvitin		minor	
30	Lipovitellin		polypeptides	
105	apo-HDL	175	apo-LDL	(Le Denmat et al., 2000)
79	apo-HDL	137	apo-LDL	
53	apo-HDL	83	γ-livetin	
46	Phosvitin	60-70	γ-livetin, apo-LDL	
32	apo-HDL	38-40	β-livetin	
		15	apo-LDL	
100	apo-HDL	75	apo-LDL	(Anton et al., 2000a)
95	apo-HDL	70	apo-LDL	
80	apo-HDL	65	apo-LDL	
54	apo-HDL	42	apo-LDL	
49	Phosvitin	35	apo-LDL	
32	apo-HDL	22	apo-LDL	
105	apo-HDL	137	apo-LDL	(Freschi et al., 2011)
79	apo-HDL	80	apo-LDL	
40	Phosvitin	65	apo-LDL	
32	apo-HDL	60	apo-LDL	
		15	apo-LDL	
		-	γ-livetin	
		-	β-livetin	
110	apo-HDL	221	apo-LDL	(Guilmineau et al., 2005)
78	apo-HDL	203	γ-livetin, apo-LDL	
47	apo-HDL	122	apo-LDL	
31	apo-HDL	93	apo-LDL	
59	Phosvitin	85	apo-LDL	
		68	apo-LDL	
		62	apo-LDL	
		55	α-livetin, apo-LDL	
		21	apo-LDL	
		20	apo-LDL	
		17	apo-LDL	
		5	apo-LDL	
110	apo-HDL	221	apovitellenin VIa	(Strixner and Kulozik, 2013)
78	apo-HDL	203	γ-livetin/apovitellenin VI	
59	Phosvitin	122	apo-LDL	
47	apo-HDL	93	apovitellenin Vb	
31	apo-HDL	85	apovitellenin V	
		72	α-livetin	
		68	apovitellenin IV	
		55	α-livetin/apovitellenin III	
		36	β-livetin	
		33	β-livetin I	
		21	apovitellenin IIa	
		20	apovitellenin II	
		17	apovitellenin I	
		5	apovitellenin Ia	

The egg yolk contains proteins with a wide range of molecular weights and pI. The applications of 2D gel electrophoresis analysis recently have been employed in identification of proteins in egg yolk. However, almost all investigations were focused on the structure of the major apoprotein present in LDL fractions and on some soluble plasma proteins (Jolivet et al., 2008; Nilsson et al., 2007, 2006). List of proteins detected in the 2D gel-Identification based on the literature are listed in Table 2.8.

Table 2.8. Protein species in egg yolk and plasma determined by 2D SDS-PAGE

Protein spots	**MW (kDa)** Theoretical	**pI** Theoretical	**MW (kDa)** Observed	**pI** Observed	**Reference**
Serum albumin (α-livetin)	67.2	5.35	65	5-5.7	(Nilsson et al., 2007)
Truncated serum albumin (aa1-aa410)	47	5.45	45	5	
Vitellogenin-1	209	9.15	-	-	
apoB,aa1-2017	224	8.68	240	8>9	
lipovitellin-1	123	9.12	130	8-9	
vitelogenin, aa1-648	72.6	9.09	75	-	
apoB,aa2136-2554	50.2	9.04	55	>9	
lipovitellin-2	26.8	10.11	28	>9	
Phosvitin	31.4	1.83	4	<4	
Ovotransferrin (Conalbumin)	75.8	6.69	80	6.5-7	(Nilsson et al., 2007, 2006)
Immunoglobulin G	60-70	6-7	65-70	6.5-8	
Glycoprotein YGP42 (β-livetin)	31.4	5.88	40	5.3-5.8	
Glycoprotein YGP40 (β-livetin)	31	6.16	35	5.5-6.3	
α-livetin	67.2	5.35	65	5-5.7	
Putative YGP30	31.46	7.55	30	-	(Jolivet et al., 2008)
Light chain of immunoglobulin	11.35	6.09	24		
Immunoglobulin G	60-70	6-7	-	-	
Serum albumin (α-livetin)	67.2	5.35	54	-	
Truncated serum albumin (aa1-aa410)	47	5.45	40	-	
Glycoprotein YGP42	31.4	5.88	44	-	
Glycoprotein YGP40	30.98	6.16	40	-	

2.4. FRACTIONATION CHALLENGE FOR PRODUCING FOLATE-ENRICHED PROTEIN EXTRACT FROM HEN EGG YOLK

Although the importance of hen egg as an economical and convenient source of metabolically active folate form has been well recognized (Seyoum and Selhub, 1998), less attention has subjected to separation of this compound. A number of different extraction methods have been employed to separate folate but many of them are based on the use of organic solvents. However, in most applications, these methods are not safe due to the possible solvent residuals in the final products (Laca et al., 2010) and furthermore the presence of food matrix may influence the bioavailability of folate (Gregory, 2001). It was shown that in the folate extract prepared from egg yolk, the presence of other egg yolk soluble components in extract has important influence on the extent of folate bioavailability in vivo. However, the degree of fat separation from egg yolk extract enhanced the concentration of folate in final egg yolk extract (McKillop et al., 2003). Therefore, there is a need to determine more efficient ways for folate separation and purification by using economical and easily scaled-up processes.

CHAPTER 3: PROBLEMATIC, HYPOTHESISAND OBJECTIVES

3.1. PROBLEMATIC

Folic acid reduces the risk of fetal neural tube defects and the level of protection increases with higher dosage. Synthetic folic acid and natural folate are converted to 5-MTHF (5-CH3-H4-folate) and this is the predominant folate form usually found in blood plasma. However, several controversies surround the potential impact of folic acid about possible masking of vitamin B12 deficiency and carcinogenic effect for consumers. Consequently, there is tendency to improve the consumption of natural form of folate. In this context, egg yolk has been recognized as an excellent source of predominant active and readily absorbable folate (5-MTHF) but negative perception of connection between high cholesterol content and the incidence of coronary heart disease affected its consumption. Moreover, an extreme increase in the consumption of extra folate from eggs is relatively ineffective since the optimal dose of 0.8-1.0mg of folate or folic acid daily for pregnant women cannot be reached. Consequently, the egg industry particularly has been requesting new technology to develop products beyond their traditional food value.

3.2. HYPOTHESIS

It is conceivable that centrifugation may offer efficient separation technique to fractionate egg yolk by means of a simple and easily scalable process. Consequently, the general hypothesis of this doctoral thesis is that it is possible to generate a folate-enriched extract from egg yolk using dilution and centrifugation technique.

3.3. MAIN AND SPECIFIC OBJETIVES

The main objective of this doctoral thesis is to establish more efficient separation method to fractionate egg yolk into granule and plasma and study the feasibility of technique in folate separation in each fraction.

Centrifugation was chosen for the separation and concentration of folate from egg yolk and scaling-up the process in order to design an integrated process for the production of a natural extract with high folate content.

Consequently, in order to achieve the main objective and verify the research hypothesis, the specific objectives of this work were:

1. To evaluate the potential of water-based method in order to separate folate in yolk fractions.

2. To investigate the potential of increasing ionic strength or applying ultrasound as pre-treatments to increase folate content of granule fraction.

3. To characterize the effects of pre-treatments (increased ionic strength, mechanical treatment: power ultrasound and high hydrostatic pressure) on the protein profile and microstructure of granules separated from egg yolk.

CHAPTER 4: Scaling-up a process for the preparation of folate-enriched protein extracts from hen egg yolks

4.1. CONTEXTUAL TRANSITION

It is conceivable that centrifugation may offer efficient separation technique to fractionate egg yolk by means of a simple and easily scalable process. But by centrifugation technique are we able to separate a yolk fraction which is enriched with folate or not. This is a question that we were trying to answer in this chapter. The work presented in this chapter has been designed in order to fractionate native egg yolk by water dilution at two scales (laboratory and pilot-scale). We will study the feasibility of fractionation techniques in folate separation in each fraction.

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The results were also presented in two posters and oral at The Functional Foods and Natural Health Products Graduate Research Symposiums (FFNHP) in the University of Manitoba (2013, 2014). Part of this research from this chapter presented in a poster entitled "Separation of hen egg yolk proteins: Mass balance and characterization of plasma and granule protein profiles in aqueous fractions" in International Food Technology (IFT) Annual Meeting & Food Expo in Chicago (IL, USA).

4.2. RÉSUMÉ

Un procédé de séparation simple a été utilisé pour fractionner le jaune d'œuf en deux parties: le granule et le plasma par une technique de centrifugation (centrifugeuse de laboratoire et à échelle pilote). Le granule à l'échelle pilote a présenté des concentrations plus élevées de protéines (66-69%) et le taux de lipides plus faible (20-26%), les sur une base sèche, par rapport à la fraction de plasma. Le bilan matière en terme de protéines, sur la matière première (jaune d'œuf), a été calculée comme étant de 96% et 89% pour les procédés de fractionnement en laboratoire et à l'échelle pilote, respectivement. Un bon accord a été atteint entre les processus de centrifugeuse de laboratoire et à échelle pilote, en termes de composition chimique et la récupération des composants de jaune d'œuf. Le plasma a gardé 66-71% des lipides à l'échelle du laboratoire et de 75-77% avec le procédé de fractionnement à l'échelle pilote. Il a été confirmé par SDS-PAGE et électrophorèse sur gel 2D que le plasma et le granule contiennent des quantités différentes de LDL et HDL et des modèles de profils protéiques différents. Des analyses HPLC ont montre que le plasma était dépourvu de folate (5-CH3-H4folate) tandis que le granule en contient trois fois plus que le jaune d'œuf.

4.3. ABSTRACT

A simple separation process was used to fractionate egg yolk into plasma and granule fractions through the use of gravitational separation with laboratory- or pilot-scale centrifuges. The granule fractions at pilot-scale presented higher protein concentrations (66–69%) and lower lipid contents (20–26%), on a dry basis, as compared to the plasma fraction. The recovery of protein, based on the raw material (egg yolk), was calculated to be 96% and 89% for the laboratory- and pilot-scale fractionation processes, respectively. Good agreement was achieved between the laboratory- and pilot-scale centrifugation processes, in terms of chemical composition and recovery of egg yolk components. The plasma fraction preserved 66–71% of the lipid with the laboratory-scale and 75-77% with the pilot-scale fractionation process. It was confirmed by SDS-PAGE and 2D-gel electrophoresis analysis that each egg yolk fraction incorporated different amount of LDL and HDL and plasma and granule fractions have different protein profile patterns. Folate content in plasma and granule fractions was measured using HPLC analysis. Folate analysis revealed that the plasma fraction of egg yolk was devoid of folate (5-CH3-H4folate), whereas the granule fraction was concentrated by a 3-fold factor in comparison to native egg yolk.

4.4. INTRODUCTION

Eggs represent an important source of nutritional and bioactive ingredients usable in the food, medical, pharmaceutical, cosmetic, and biotechnological industries (Anton, 2007c). Egg proteins are highly digestible, containing essential amino acids in a profile that is comparable to the ideal balance of amino acids needed by humans. Eggs also supply various minerals and major vitamins. The hen egg has been also considered as an excellent natural source of folate (Strandler et al., 2011), contributing 10–20% of the daily requirement for folate (Song and Kerver, 2000).

The ability of the egg to be used as functional food has been established (Surai and Sparks, 2001). Enrichment of eggs with anti-oxidants and vitamins has been well documented (Sahlin and House, 2006; Surai and Sparks, 2001).

With respect to the water-soluble vitamin folate, recent studies have revealed that the folate content of eggs can be enhanced due to the hen's ability to convert folic acid from fortified feed to natural forms (Hebert et al., 2005; House et al., 2002) with high bioavailability (House et al., 2003b; Sugiyama et al., 2012). Folic acid, the synthetic form of folate vitamin, either from supplements or fortification, has been shown to reduce the incidence of neural tube defects in pregnancies (MRC, 1991; Ray et al., 2002). However, an excess of the synthetic form of folate for the population as a whole may not necessarily be beneficial (Smith et al., 2008). There are several studies that seem to indicate a potential risk of excess synthetic folic acid intake (Mason, 2011; Wien et al., 2012). One study showed an apparent increase in the incidence of colorectal cancer after the mandatory fortification of folic acid in both the USA and Canada (Mason et al., 2007). Conversely, the native dietary forms of folate in physiological amounts are known to have no adverse effects. Egg yolk has been reported to contain 87–100% of folate in the form of 5-CH3-H4folate as the most abundant form of folate (Seyoum and Selhub, 1993; Strandler et al., 2011; Vahteristo et al., 1997), with folate principally restricted to the yolk fraction. According to recent studies (Strandler et al., 2011), one egg (60 g) provides 40–50 μg of folate which corresponds to 10–15% of

the recommended daily allowance of 400 μg for adults or to 13–16% of the estimated average requirements of 320 μg/day (Medicine and DRIs, 1998).

Egg yolk is a very complex biological system consisting of non-soluble protein aggregates (granules) in suspension in a clear yellow fluid (plasma) that contains low-density lipoproteins (LDLs) and soluble proteins (Anton, 2006). Classical separation methods of egg yolk have utilized solvents (Kwan et al., 1991), or conditions which in turn caused the protein fractions of egg yolk to denature (Liot, 2002). However, in order to preserve the techno-functional properties of egg yolk fractions, the native behavior of egg yolk must remain unaffected. Egg yolk can also be fractionated into two fractions of plasma and granules by dilution followed by centrifugation. Previous studies have relied on fractionation processes based on discontinuous lab scale methods (Mc Bee and Cotterill, 1979), where centrifugation at high g-forces (10,000×g) and long residence times (45 min) is utilized. In recent studies, the discontinuous fractionation methods with long residence times have been applied to determine the specific functional properties of granule/plasma-fractions where these methods provided the advantage of high separation efficiencies (Laca et al., 2011, 2010; Strixner and Kulozik, 2013) in separation of the fat and aqueous fractions of egg yolk. Studies by Laca et al. (2011, 2010) resulted in a granule fraction that contained 34% lipid and 60% protein.

However those methods utilized different strategies (e.g. pH adjustment, the use of acids, bases and/or salts with extreme water dilution) which all have disadvantages such as complexity of procedures, or compatibility for ultimate use as a food or medicinal ingredient.

The use of centrifugation steps offers high clarifying potential and throughputs (Lander et al., 2005) for the separation of biological products, as the volume of the particles or the density gradient between the particles and the liquid are small (Spelter et al., 2010).

The tubular bowl centrifuges are well established efficient unit operations in the field of biotechnology (Stahl et al., 2008) and they offer the highest centrifugal forces (Lander et al., 2005) and can be used to scale-up an egg-yolk fractionation procedure.

The objective of the present study was to develop and scale-up a sequential separation method using water to fractionate native hen egg yolk for the preparation of a folate-enriched protein extract. The present work was based on the hypothesis that folate would be found in higher proportion in the protein fraction (granule). The composition of fractions generated from laboratory-scale and pilot processes were characterized and mass balances were determined.

4.5. MATERIALS AND METHODS

4.5.1. Hen eggs

Fresh white shelled eggs of weight class M (53–56 g) or L (58-63 g) were purchased from a local supermarket and stored in a cooling chamber at 4°C until further use.

4.5.2. Chemicals

All chemicals, unless specified otherwise, were obtained from VWR (Quebec, Canada). Acetonitrile was of HPLC gradient grade; other chemicals were of analytical quality. Water was purified (≤0.1 μS cm^{-1}) using a Milli-Q system (Millipore, USA). Standard for HPLC method; 5-Methyltetrahydrofolic acid (5-CH3-H4folate) disodium salt was from Sigma-Aldrich (Wisconsin, USA). The standard folate was purchased in lyophilized form and was stored at -20°C until use. The stock solutions of folate for HPLC, 50 nM, were prepared in ascorbate buffer (20 g/L sodium ascorbate; 12.1 g/L Trizma base; pH 7.8). The calibration solutions were prepared immediately before use by diluting the stock solution with extraction buffer (20 g/L sodium ascorbate; 12.1 g/L Trizma base; pH 7.8).

4.5.3. Fractionation of egg yolk

Each egg was weighed, and then yolks and albumen were separated manually and the weights were registered. The residual albumen and chalazae were eliminated manually

from the yolk using blotting paper. The removal of the vitelline membrane was achieved using tweezers. Next, the yolk material was diluted by mixing with Milli-Q water (1:1 v/v) and gently homogenized by stirring with a glass rod in a beaker cooled in an ice bath.

4.5.3.1. Lab scale process

The laboratory-scale fractionation method was developed as a modification of the procedure described by (Laca et al., 2010). All experiments were performed on three separate batches by choosing one dozen eggs for each batch.

The egg yolk fractionation studies were performed by using a RC-5B PLUS centrifuge (SORVALL® Superspeed®, Newtown, CT, USA) and a fixed-angle rotor (SS-34) by applying the conditions of 10,000g at 4°C for 45 min. Recent findings have shown the absence of granule-particles in plasma and the complete separation of granule and plasma with centrifugation at 10,000×g (Strixner and Kulozik, 2013). As a result, a g-force of 10,000×g was chosen for the lab-scale study. Diluted fresh egg yolk material was placed in centrifugation tubes (50 mL) and filled to capacity. Following centrifugation, the tubes were decanted slowly to separate the plasma and granule fractions. The deep orange yellow supernatant stream was defined as the plasma and the sediment was defined as the granular fraction. Both fractions were stored over night at 4°C for further investigations. All experiments were performed in triplicate.

4.5.4. Pilot scale process

All experiments were performed on three separate batches, with two dozen large size eggs per batch. The fractionation studies were performed on a tubular bowl centrifuge (CEPA Centrifuge, series LE, Germany) with a maximum centrifugal force of 40,000×g. The tubular bowl centrifuge (Fig. 1) is equipped with (a) a cylinder type SK which is a serum clarifying cylinder, and the suspension is then led by (b) a feed pump from below over the (c) nozzle holder with a screwed on nozzle. The liquid (diluted egg yolk) is injected into the cylinder and cleaned continuously by the centrifugal force. The cleaned or separated liquid

is collected at the outlet aperture at the cylinder head into the (d) collecting trays and is drained off via the apertures of the collecting trays.

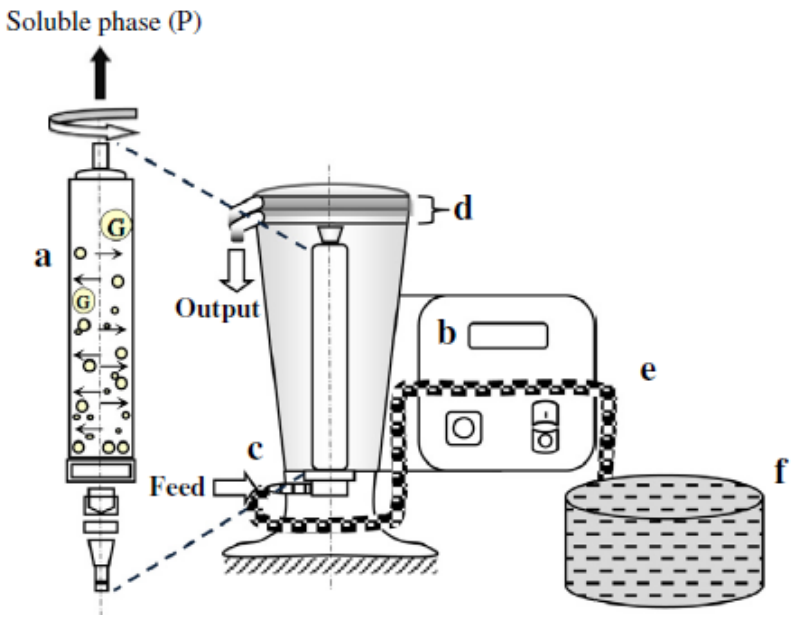


Figure 4.1. Schematic of the tubular bowl vertical centrifuge (CEPA Centrifuge, series LE, Germany) used for pilot-scale experiments. Cross-sectional views: (a) cylinder type SK, (b) feed pump, (c) nozzle, (d) collecting trays, (e) tube, and (f) feeding tank. P: Plasma, G: Granule.

The cylinder type SK which was used in this experiment operates continuously with a speed revolution of 15,000–40,000 min^{-1}, with a throughput capacity of 30 l/h and usable content of 0.25 dm^3.

Diluted fresh egg yolk was pumped continuously to the tubular bowl centrifuge. Separation of egg yolk was achieved at a maximum centrifugal force. Going from laboratory-scale process to the pilot-scale production, the maximum centrifugal force of 40,000×g was adopted because it provided maximal equipment feeding capacity and granule compaction. The geometry of the tubular bowl centrifuge is similar to that which was successfully used in the separation of biological products (Spelter et al., 2010) and the best results with less product loss were achieved at the highest centrifugal force. The clarified plasma was expelled from the top of the centrifuge chamber through a collection tray (d) while the sedimented granule was collected in the tubular bowl cylinder (a) and formed a paste. The retained granule cake was dry and densely packed by the high g-forces of centrifugation during the pilot-scale separation. The removal of the sediment required a dismantling of the

rotor and the bottom part of the rotor itself. Both fractions were stored at 4°C after the fractionation process to permit further investigations and analyses.

4.5.5. Chemical analyses

The dry matter (DM) content of the homogenized egg yolk, plasma and granule fractions was determined by drying samples at 110°C in a vacuum oven for at least 14 h, and the DM content was expressed as g dry matter per 100 g fresh sample.

The total lipid content determination was based on the official AOAC method (AOAC, 2005).

The total protein content of egg yolk and its fractions was determined with a nitrogen gas analyzer system (LECO FP-528, Model 601-500, LECO Co., USA). A conversion factor of 6.25 was used to convert total nitrogen values into protein.

4.5.6. Extraction and analysis of folate content

A reversed-phase high-performance liquid chromatographic (HPLC) method with fluorescence and ultraviolet detection was used to detect and quantify the most abundant folate (5-CH3-H4-folate) in yolk fractions. Calibration was performed with 5-CH3-H4-folate standard and the correlation coefficient for the calibration curve was $r \geq 0.998$.
The extraction of folate from egg yolk fractions (plasma and granule) was done immediately following the fractionation process, and the HPLC analyses were carried out using fresh samples, given that folates are susceptible to degradation (McKillop et al., 2003). Folates were extracted from egg yolk fractions using the method described by House et al. (2002) with minor modifications.

Portions of the samples (egg yolk 0.5 g, plasma 1 g, and granule 1 g) were weighed in triplicate in plastic centrifuge tubes, and were mixed with 10 mL extraction buffer (20 g/L sodium ascorbate; 12.1 g/L Trizma base; pH 7.8). Tubes were placed in a boiling water

bath for 60 min for extraction, and then rapidly centrifuged at 3000g for 35 min at 4°C. Supernatants were collected and retained.

A sample from each flask was filtered in 0.2 μm Nylon membrane and subjected to HPLC analysis. HPLC analysis was performed using an Agilent 1100 HPLC system (Agilent Technologies, Inc., Palo Alto, CA) equipped with a G1322A degasser, a G1312A binary gradient pump, a G1329A autosampler and a 474 fluorescence detector from Waters (Milford, MA). The software Agilent Chemstation was used to control the HPLC system, and to collect and process the chromatographic data. The separation of folates by HPLC involved the use of a Kinetex™ 2.6 μm XB-C18 100 Å; 75× 2.10 mm column. The following conditions were employed: column temperature, 23°C; flow-rate, 0.4 mL/min; injection volume, 1 μL; fluorescence detection, 290 nm excitation and 360 nm emission; UV detection, 290 nm. The mobile phase employed a binary gradient mixture of 30 mM potassium phosphate at pH 2.3 and acetonitrile. The gradient started at 10% (v/v) acetonitrile and rose to 28% within 5 min. Quantification of folate was based on the external standard method. The 5-CH3-H4folate was used for spiking and the recovery (R) was calculated according to the following equation (Strandler et al., 2011):

%R = [(CSpiked sample - C Unspiked sample)/CStd spiked to sample]×100

Equation 4.1

where C is concentration and Std is the standard (5-CH3-H4folate) added to the folate extract sample.

Recovery tests performed by spiking at two different concentrations in the yolk samples showed good recovery for 5-CH3-H4folate with recoveries of 102.7% (±22.5) and 100.2% (±5.77) for the laboratory- and pilot-scale processes, respectively. The recovery of 5-CH3-H4folate from the granule samples was calculated to be 103.28% (±1.01) and 106.34% (±8.60) for the laboratory- and pilot-scale process, respectively.

4.5.7. Performance parameters of egg yolk fractionation

A mass balance was calculated for the various components in order to compare the recovery (%) at lab- and pilot scale (Equation 4.2):

Recovery (%) = $[(DM_{mass})_P + (M_{mass})_G / (DM_{mass})_Y]100\%$ Equation 4.2

where subscripts Y, P, and G refer to yolk, plasma and granule, respectively.

From a complete key component mass balance on all processes other parameters can be calculated:

R_i (%) = $[(C_{iP} \times V_{iP} + C_{iG} \times V_{iG}) / (C_{iY} \times V_{iY})] \times 100$ Equation 4.3

where i is the total solid, protein, lipid, and folate, R is the recovery, V is the volume of material in the respective phase and C indicate concentration.

The concentration effect of the egg yolk fractionation process was determined by calculating a folate concentration factor (CF):

CF = $[Folate]_{Granule} / [Folate]_{Yolk}$ Equation 4.4

These parameters were used to compare laboratory- and pilot-scale centrifugation approaches.

4.5.8. Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

All SDS-PAGE analysis was performed by using commercially available precast linear gradient polyacrylamide gels (4–20%; Bio-Rad, Canada). Specifically, 1% protein solution were prepared and left overnight, followed by dilution with deionized water so that the amount of proteins loaded onto the gel was approximately 20 µg. Thereafter, samples were diluted 1:1 (v/v) in a dissociation buffer consisting of a 0.5 M Tris–HCl pH 6.8, 0.5% bromophenol blue, 35% glycerol, 5% b-mercaptoethanol, 10% (w/v) SDS solution, and heated in boiling water for 5 min. The samples treated under reducing conditions were then centrifuged at 10,000×g for 5 min, and 10 µL of the supernatant were transferred into each well. Electrophoretic separation was performed in a Multiphor II system applying a maximum voltage of 90 V and a current gradient increasing from 15 to 50 mA with a migration buffer consisting of a 0.02 M Tris (hydroxymethyl) aminomethane, glycine 5 M, SDS (w/v) 0.1% solution. The proteins were stained using standardized protocols (Coomassie blue 0.1%, methanol 50%, acetic acid 10% and water 40%). The gels were destained in a solution containing acetic acid (10%), methanol (40%) and water (50%). For the detection of phosvitin, which is not stained by Coomassie blue (Hegenauer et al., 1977), 0.1 M aluminum nitrate was added to the staining solution (Guilmineau et al., 2005).

4.5.9. Two dimensional electrophoresis

Protein profiles were further characterized using two-dimensional electrophoresis according to standard protocols (Gorg et al., 2000). Isoelectric focusing (IEF) was carried out at 20°C with an IPGphor system (version A2, 1998, GE Healthcare Life Sciences) using 13 cm Immobiline™ DryStrip gel covering a pH range of 3-10 (GE Healthcare Life Sciences). All samples (500 µg protein/250 µL) were cup-loaded onto the strip. Analytical gels were consecutively stained with the usual staining procedure (Coomassie blue 0.1%, methanol 50%, acetic acid 10% and water 40%). The gels were destained overnight in a solution containing acetic acid (10%), methanol (40%) and water (50%). For the detection of phosphorylated proteins 0.1 M aluminum nitrate was added to the staining solution based on the method explained by Guilmineau et al. (2005).

4.5.10. Statistical analysis

At least three replicates of each fractionation process and measurements were carried out. Statistical analyses were performed using t-test and one-way analysis of variance (ANOVA, $p < 0.05$) by means of the statistical package Minitab Statistical Software release 16.2.3 for Windows (Minitab®, State College, PA). Standardized skewness and standardized kurtosis were used to assess normality.

4.6. RESULTS AND DISCUSSION

4.6.1. Overall composition of the fractions

Table 4.1 shows the composition of the obtained fractions compared with the original yolk composition. The main components of native egg yolk are lipids (about 57–62 g/100 g of the dry matter; Table 4.1) and the lipid to protein ratio has been reported to be approximately 2:1 (Anton, 2007a). As can be seen, the plasma fraction constitutes 22.36 g/100 g and granule 45.47 g/100 g of initial yolk dry matter at laboratory-scale centrifugation.

At pilot-scale separation of egg yolk, the dry matter of plasma fraction remained unchanged for about 22.36 g/100 g whereas the dry matter of granule fraction was significantly different from granules separated at lab-scale. The dry matter content in the granule fraction increased from 45.47 g/100 g in the lab-scale method to 51.39 g/100 g for the pilot scale separation, reflecting differences in the efficiency of separation.

According to the data in Table 4.1, the granule fractions represent the higher protein and lower lipid constituents of the egg yolk. Granules separated by the pilot-scale method contained 11.83 g/100 g lipid and 35.05 g/100 g protein which represented 23% lipid and 68% protein on a dry matter basis. However, a higher concentration of lipid (13.91 g/100 g) and a lower concentration of protein (27.61 g/100 g) were measured for granules separated by laboratory-scale centrifugation, equating to 32% lipid and 58% protein of granule dry matter. Calculations from these data show that, with the centrifugation method used in the pilot-scale process, the protein:lipid ratio was 3:1, whereas it was 2:1 for the lab-scale process, and both techniques yielded ratios that differed from the 1:2 protein:lipid ratio found in the native egg yolk. However, the protein content of the pilot-scale produced granule extract was found to be more concentrated than the corresponding egg yolk.

The high protein and the low lipid contents of granules are due to a high percentage (70%) of high density lipoproteins (HDL) in this fraction (Burley and Cook, 1961). These

lipoproteins consist of 80% proteins and only a small quantity of lipids (Cook and Martin, 1969b).

During pilot-scale fractionation, a higher value of 16.55 g lipid/100 g was observed for the plasma fraction compared with 14.05 g lipid/100 g in the plasma fraction derived through the lab-scale centrifugation process. At the dilution conditions employed, the plasma fraction of egg yolk contains most of the egg yolk lipids, accounting for approximately 71–76% on dry basis. As documented in Table 4.1, the composition of the plasma fractions derived from the pilot-scale process contained significantly less protein and more lipid than the fraction derived from the lab-scale process. The higher content of lipid in plasma is generally associated with low density lipoproteins (LDL) which represented a high proportion (89%) of the lipids in egg yolk (Burley and Cook, 1961). The composition of the lab-scale fractions agrees with that presented by Laca et al. (2010) and Li-Chan et al. (1995).

4.6.2. Folate enrichment

The folate content of egg yolk and the respective fractions (plasma and granule) from both the laboratory- and pilot-scale processes are presented, on a wet and dry basis, in Table 4.1. The average folate content of egg yolk was quantified as 203.84 (±12.55) μg 100 g^{-1} yolk sample and 314.9 (±143.5) μg 100 g^{-1} yolk sample for lab- and pilot-scale separation process, respectively. Although the results for egg yolk appear to be lower in laboratory-scale process, results showed no significant difference in ANOVA evaluation. The variation in the folate content of native egg yolk may be due to the size of the eggs or the fact that different egg producers distribute eggs to the market, and the hen feeding conditions may vary across production facilities. Similar observations were reported in the study of Strandler et al. (2011), Dickson et al. (2010) and Hebert et al. (2005) where different conditions at the farms and/or variation between different strains of the breed resulted in a large standard deviation. These data are based on the folate content in eggs from the market that were of medium and large sizes. The data are in good agreement with (Strandler, 2012; Strandler et al., 2011) who measured the average of 252 (±49) μg 100 g^{-1} folate in raw egg

yolk (ranging from 195 to 382 μg 100 g^{-1} folate content in egg). However the slight difference is likely to be related to methodological differences where the aforementioned researchers used different extraction method to separate folate from egg yolk. (Strandler, 2012; Strandler et al., 2011) have used different extraction buffer with moderate heat treatment (shorter time and lower temperature). The folate content in the granule fraction of egg yolk derived from the lab-scale process amounted to 670 (±35) μg 100 g^{-1} of granule sample compared to 1030 (±286) μg 100 g^{-1} of granule sample observed in the granule fraction extracted via the pilot-scale process. Folate was not detected in the egg yolk plasma fractions derived from either process (Table 4.1).

Table 4.1. Composition of egg yolk and fractions from lab- and pilot-scale processes.

Composition	Sample	Lab scale		Pilot scale	
		Wet wt.	DB[1]	Wet wt.	DB
Dry matter (g/100 g)	Egg yolk	50.88 ± 0.27	-	50.54 ± 0.52	-
	Plasma	22.36 ± 1.15	-	22.36 ± 1.92	-
	Granule	45.47 ± 0.74[a]	-	51.39 ± 1.39[b]	-
Protein (g/100 g)	Egg yolk	16.15 ± 0.19	31.84 ± 0.40	15.24 ± 0.37	30.15 ± 0.37
	Plasma	4.63 ± 0.19[a]	21.84 ± 0.60	4.20 ± 0.36[b]	18.83 ± 1.26
	Granule	27.61 ± 0.35[a]	57.62 ± 9.17	35.05 ± 1.57[b]	67.64 ± 1.63
Fat (g/100 g)	Egg yolk	28.65 ± 1.41	56.50 ± 2.85	31.52 ± 0.77	62.37 ± 0.93
	Plasma	14.05 ± 0.96[a]	70.61 ± 5.90	16.55 ± 0.61[b]	75.71 ± 1.00
	Granule	13.91 ± 0.57[a]	31.70 ± 1.04	11.83 ± 1.17[b]	23.01 ± 2.79
5-CH3-H4folate (µg/100 g)	Egg yolk	203.84 ± 12.55[a]	399.31 ± 9.75	314 ± 143.5[a]	657.72 ± 151.2
	Plasma	ND[2]	-	ND	-
	Granule	670.05 ± 34.82[b]	1474.41 ± 41.78	1029.7 ± 286.1[c]	2078.8 ± 307.4

Experiments were repeated on three separate occasions, and values are mean ± SD.

[a–c] Different letters in columns (lab-scale and pilot-scale) refer to significantly different values as calculated by ANOVA.

[1] Calculations on dry matter basis.

[2] ND: Not Detected.

The degree of concentration of folate in the granule fraction as a result of pilot-scale preparation may depend to an extent on the water content of the starting material. Further, it was demonstrated that, in egg yolk, the removal of fat greatly enhanced the concentration of folates in the final egg yolk extract (McKillop et al., 2003). This is in good agreement with presented results showing significantly greater folate yield from granule fractions, indicating that most of the fat content of the yolk is retained in the plasma fraction and 100% of the folate is located in the granule fraction. The results of our protein study revealed different protein profiles for the granule and plasma fractions. Since folate was located in the granule fraction, we can assume that the folate might be associated with select proteins found in the granule fraction. However, there is no evidence which would conclusively point to this protein as being a putative folate-binding protein (Sherwood et al., 1993).

The folate extraction concentration factor (CF) was calculated to be 3.29 and 3.28 for the laboratory- and pilot-scale separation processes, respectively. The folate content of egg yolk (399.31 μg 100 g^{-1} of yolk sample and 657.72 μg 100 g^{-1} of yolk sample) and granule fraction (1474.41 μg 100 g^{-1} of granule sample and 2078.8 μg 100 g^{-1} of granule sample) was calculated based on a dry matter basis, and presented in Table 4.1 for both the laboratory and pilot-scale separation processes, respectively. The CF values indicated that the yolk granules extracted by centrifugation methods contain approximately 3-fold higher folate content compare to the native egg yolk. The capacity of granules fraction of yolk in maintaining almost all folate content of egg yolk can potentially add more value to other verified techno-functional properties of the granule, e.g. lower cholesterol content (Laca et al., 2010; Orcajo et al., 2013) compared to yolk, and good emulsion stabilizer and gelling properties (Anton, 2013; Laca et al., 2010). These results could favor application of granule fraction as substitute for yolk in food industry.

4.6.3. Protein profile of fractions obtained at lab- and pilot-scale

The protein compositions of the fractions were analyzed by SDS-PAGE in order to characterize the protein constituents of the granule and plasma extracts separated through the fractionation processes. The results of the protein profiling by SDS-PAGE, using aluminum nitrate staining, for egg yolk and the respective fractions (plasma and granule) are shown in Figure 4.2. The use of aluminum nitrate permitted the additional staining of phosvitin. The electrophoresis of egg yolk protein has been generally conducted under reducing (R) conditions (e.g. addition of 2-mercaptoethanol to the dissociation buffer) (Calvo et al., 1993; Tolkach and Kulozik, 2005) but it was reported that the induced dissociation of disulfide bridges and structural modifications (uncoiling) can modify the electrophoretic mobility (Guilmineau et al., 2005). Therefore, in our study we employed both reducing and non-reducing conditions. Lane 1 (Figure 4.2.) shows the protein composition of native egg yolk, where the bands were identified based on their molecular weight and by comparison with the proteins identified by (Guilmineau et al., 2005). The egg yolk profile contains 20 bands having a MW ranging from 5 to 221 kDa (Figure 4.2., lanes 1 and 8). Different SDS–PAGE patterns were obtained for the two fractions. Differences between the distributions of the bands in each fraction were visually apparent. Five major bands were identified in the protein profile of the granule fraction (Figure 4.2., lanes 3 and 6), with the bands having an apparent molecular weight between 31 and 110 kDa. The heaviest proteins of the yolk are concentrated in the granule fraction. Granules contain HDL apoproteins (β- and α-HDL), phosvitin (β- and α-phosvitin) and apovitellin. The MW of HDL apovitellins were measured at 110, 78, 47, and 31 kDa, and these values are consistent with the values reported by (Anton et al., 2000b), Guilmineau et al. (2005) and recently by Strixner and Kulozik (2013). We used the nomenclature system of Strixner and Kulozik (2013) to name the major apovitellins. The profile of apovitellins under R and NR conditions did not differ (Figure 4.2., lanes 3 and 7). In the work of Guilmineau et al. (2005), the authors measured a single band at 59 kDa for phosvitin, while Jiang and Mine (2000) showed a single band with an apparent MW of 35–36 kDa for both commercial and laboratory-prepared phosvitin. In our study, however, we measured double bands at 40 kDa for α-phosvitin and 45 kDa for β-phosvitin in the same position as that observed with commercially purified phosvitin (Figure 4.2., lane 9). Our observations are in agreement with those reported in previous studies (Anton, 2007c; Castellani et al., 2006; Causeret et al.,

1991; Ko et al., 2011) where two isolated bands were characteristic of α- and β-phosvitin. The electrophoretic mobility of phosvitin was not affected by R conditions. This was explained by the absence of cysteine residues in the molecule and the lack of formation of intra- or intermolecular disulfide bridges (Boye et al., 1997; Guilmineau et al., 2005). The protein profile of plasma fractions revealed the presence of nine bands (Figure 4.2., lanes 2 and 7) corresponding to LDL apoproteins and livetins. The bands possessing apparent MWs of 203, 122, 73, 68, 55, 36, 33, 17, and 5 kDa corresponding to LDL proteins were located in the plasma profile. Livetins were identified with apparent MWs of 73, 55, 36, and 33 kDa under NR conditions, however it was reported that band 203 kDa contains γ-livetin in addition to apovitellenin. A slight shift in the MWs of livetins was observedunder R conditions. This corresponds to the presence of intramolecular disulfide bonds in these proteins (Guilmineau et al., 2005).

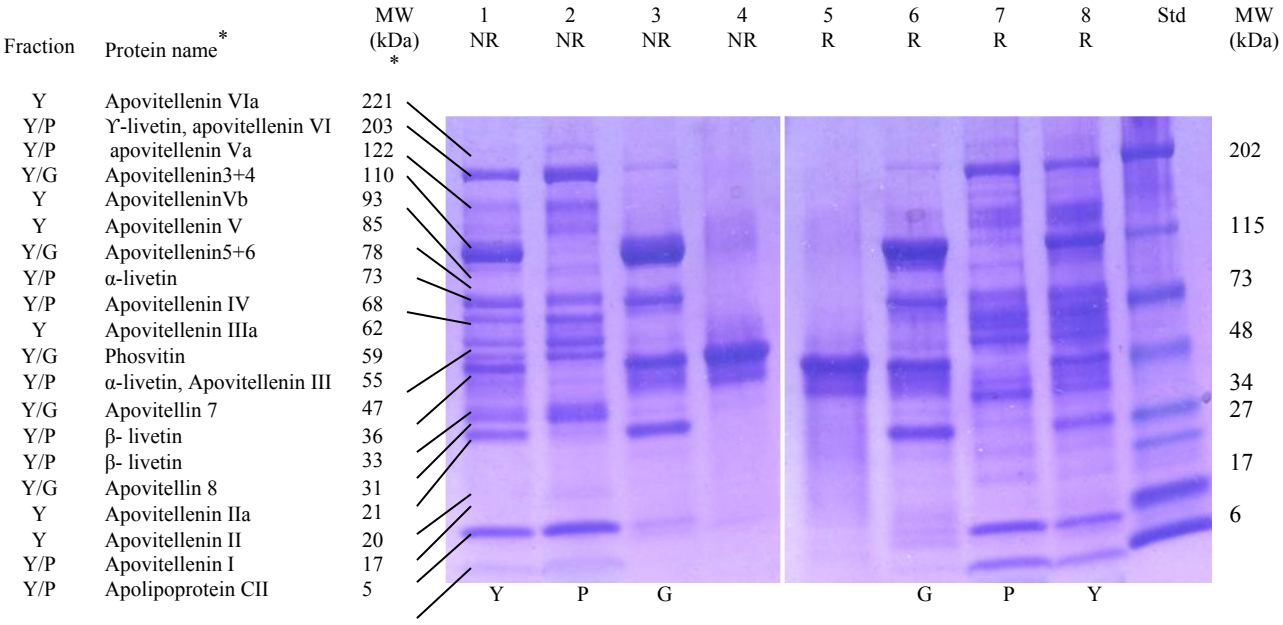
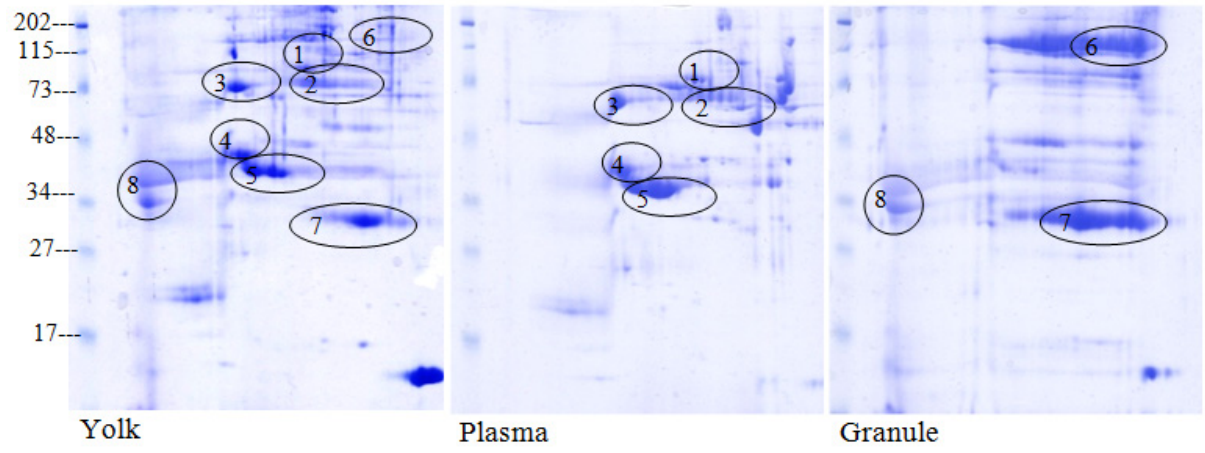


Figure 4.2. SDS-PAGE profile of proteins of EY fractions in NR and R conditions: (1 and 8) whole EY; (2, 7) Plasma fraction; (3, 6) Granule fraction; (4 and 5) commercial phosvitin; (Std) MW standard. The volume of 20µl of proteins were placed in each well.
*All protein lanes are identified and named according to Guilmineau et al. (2005).

By comparing the protein profile of egg yolk fractions in laboratory- (data are not shown) and pilot-scale separation processes, it was observed that the intensity of protein bands did not change. Based on gel electrophoresis analysis, plasma and granule fractions have different protein composition. This indicates that HDL and LDL proteins have different amount of lipid or LDL constituents incorporated within their structure.

These observations were further confirmed by the results of the two-dimensional (2D) polyacrylamide gel electrophoresis (Figure 4.3.). Numerous spots (p*I* and MW) were detected with changes in intensities in the protein profiles of the plasma and granule fractions. 2-D PAGE pattern of plasma and granule fractions were compared with that of native egg yolk (Figure 4.3.). The spot locations (p*I* and MW) of the plasma proteins were considerably different from those of granule proteins. The most predominant protein bands (marked by ring) were identified by Nilsson et al. (2007, 2006) and can be seen in the 2-D PAGE of whole egg yolk. The major proteins ovotransferrin (conalbumin), lipovitellin I, immunoglobulin G (γ-livetin), serum albumin (α-livetin), yolk plasma glycoprotein (β-livetin), lipovitellin-II, and phosvitin. According to (Nilsson et al., 2006) five groups of spots (α-livetin, γ-livetin, β-livetin and ovotransferrin) were identified in plasma protein profile, the MW of the proteins range from about 35 to 80 kDa, and p*I* values vary between 5 and 8. The appearance of very distinct chain of multiple spots in each group was related to different degrees of phosphorylation (Nilsson et al., 2007). The most abundant proteins of granule fraction comprised vitellogenin-derived lipovitellins (Figure 4.3., spots 6 and 7), apolipoprotein (not detected) and phosvitins (Figure 4.3., spot 8). The location of phosvitin, which is only visible when the gel is stained in the presence of aluminum nitrate, is in agreement with observations of Nilsson et al. (2007). In our studies, the gels were stained in the absence and presence of aluminum nitrate to avoid interference with the staining of other protein spots. By staining phosphorylated peptides through the addition of 0.1 M aluminum nitrate in Coomassie blue, more spots were visualized in the granule fraction of egg yolk. The spots at acidic pH (3-4) and in the vicinity of molecular weight of 30-35 kDa, which were visualized in the granule fraction as well, were identified as phosvitin. The major and most characteristic proteins of egg yolk has been recognized as lipovitellins, phosvitins, and yolk plasma glycoprotein (van het Schip et al., 1987; Yamamura et al., 1995) which were distributed in the two fractions of egg yolk through the conditions we applied in the

fractionation processes, and they were clearly apparent in both 1D and 2D SDS-PAGE. However, to date, many of the proteins located in the granule fraction of egg yolk remain uncharacterized. Many studies which have applied high-resolution techniques for proteome analysis, including SDS-PAGE and 2D gel electrophoresis, have chosen either whole egg yolk (Nilsson et al., 2007) or yolk plasma (Nilsson et al., 2006) for protein identification, however granular proteins (phosvitin, LDLs) have generally been excluded (Raikos et al., 2006).



Spot	Location	Protein	MW (kDa)	p*I*
1	Y/P	Ovotransferin (conalbumin)	80	6.5-7
2	Y/P	Immunoglobulin (ϒ-livetin)	65-70	6.5-8
3	Y/P	α-livetin	65	5-5.7
4	Y/P	YGP42 (β-livetin)	40	5.3-5.8
5	Y/P	YGP40 (β-livetin)	35	5.5-6.3
6	Y/G	Lipovitellin-I	130	8-9
7	Y/G	Lipovitellin-II	28	>9
8	Y/G	Phosvitin	35	<4

Figure 4.3. Two-dimensional polyacrylamide gel of whole egg yolk, plasma and granule fraction of proteins. The most prominent protein bands, marked by rings, were identified and named according to (Nilsson et al., 2007, 2006). The molecular sizes of the marker are given in kDa. Gels were stained by adding 0.1M aluminum nitrate into Coomassie blue. Granule and plasma fractions were separate from yolk by tubular bowl centrifuge (40,000×g).

4.6.4. Mass balance and centrifugation performance

Taking into account the weight of the liquid egg yolk streams and the concentrations of their main components (Table 4.2.), the masses of the recovered components were calculated. The total solid material recovered after the fractionation processes were 50 g (97%) and 140 g (91%) for the lab- and pilot-scale processes, respectively. Although, there was no significant difference between the lab- and pilot-scale processes, the material balances revealed that 10% of the final material was lost during the pilot-scale extraction process, compared with 8% losses in the lab-scale centrifugation process. The recovery of folate in granule fraction based on the starting raw material (egg yolk) calculated and stated as 89% and 84% in laboratory- and pilot-scale separation, respectively (Table 4.2.). The protein content and protein yield (YP) for each fraction were calculated and are summarized in Table 4.2. The fractionation at lab-scale resulted in a higher YP in granule extract (7 g/100 g protein) than plasma (5 g/100 g protein). The YP of 21 g/100 g protein was calculated for both granule extract and plasma. However, the recoveries of proteins based on the raw material (egg yolk) were calculated to be 96% and 89% for the laboratory-scale and pilot-scale fractionation processes, respectively. The low protein recovery achieved with the pilot-scale process was likely due to the loss of material experienced when expelling the final product from the centrifuge cylinder.

The distribution of lipid in the plasma and granule fractions for both the laboratory- and pilot-scale processes is presented in Table 4.2. The yield of lipid (YL) calculated in plasma fractions was higher than observed in the granule fraction (Table 4.2) for both the laboratory- and pilot-scale processes, with 76% and 84% of the lipid located within the plasma derived from the laboratory and pilot-scale fractionation processes, respectively. The total mass of lipid recovered was 94% and 92% of the initial content of egg yolk for the laboratory- and pilot-scale processes, respectively.

Table 4.2. Mass balance upon centrifugation at lab-, pilot-scale and recovery (%) of folate, protein and fat after fractionation process.

		Weight (g) in DM	Recovery (%)	Recovered folate (%)	Yield of protein (g/100 g wet weight)	Recovered protein after fractionation (%)	Yield of lipid (g/100 g wet weight)	Recovered lipid after fractionation (%)
Laboratory process	Yolk[a]	51.30 (±0.34)			11.83 (±3.67)		20.98 (±6.53)	
	Plasma	36.81 (±0.11)			5.35 (±1.86)		16.17(±5.20)	
	Granule	13.00 (±1.15)			6.96 (±1.84)		3.49 (±0.82)	
	Total		97.08 (±3.19)	89.37 (±5.99)		96.03 (±4.35)		93.81 (±1.84)
Pilot-scale process	Yolk[a]	154.15 (±18.87)			46.53 (±6.30)		96.03 (±10.47)	
	Plasma	109.49 (±10.55)			20.68 (±3.02)		81.22 (±9.94)	
	Granule	30.81 (±5.06)			20.95 (±3.94)		7.03 (±0.53)	
	Total		91.10 (±1.22)	84.48 (±31.52)		89.25 (±2.88)		91.86 (±2.67)

[a] Fresh homogenized egg yolk. Experiments were repeated on three separate occasions, and values are mean ± SD of N = 9.

Operation of the bowl centrifuge in batch mode gave comparable though slightly lower recovery values at the pilot-scale, which is likely the result of solids lost during manual collection of the granules with the pilot-scale process.

Table 4.3 shows the compositional characteristics of granule extracts compared to egg yolk. The ratio of the protein concentration into total solid content of initial product (egg yolk) and granule extracts, separated by the laboratory- and pilot-scale processes, were calculated to be 30%, 52%, and 72%, respectively. These data show that we are able to obtain a fraction with a protein concentration almost two times higher than native egg yolk which is also low in lipid content. However, the calculated ratio of protein content to total soluble solids was improved by 10% through the use of a pilot-scale centrifuge with higher speed. Additionally, the extraction conditions employed led to an increase in the ratio of folate to protein in the granule extracts when compared to the native egg yolk (Table 4.3). The increased ratio of protein to total soluble solids in the granule fraction at pilot-scale separation compare to lab-scale separation was in accordance with the ratio of folate/protein granule extracts. The folate is likely protein bound however the nature of this protein remains to be conclusively determined (Sherwood et al., 1993).

Table 4.3. Compositional characteristics of granule extracts compared to egg yolk.

Separation process	Protein (g/100 g solids)	[Folate] (lg/protein (g))	Folate concentration factor (CF)
EY	30	13	-
G_{Lab}	52	24	3.29
G_{Pilot}	72	29	3.28

4.7. CONCLUSION

Our study shows that good agreement was achieved between the laboratory- and pilot-scale (tubular bowl) centrifuge in terms of chemical composition and recovery of egg yolk components. Folate analysis revealed that the plasma fraction of egg yolk was devoid of 5-CH3-H4folate, whereas the granule fraction was concentrated by a 3-fold factor in terms of 5-CH3-H4folate in comparison to native egg yolk. Considering the recommended daily intake of 400 µg folate for adults, the granule extracted from one egg (58–63 g) by our lab- and pilot separation method can provide 7–8% of daily requirement. More work will be needed to fully characterize the distribution of lipid fractions, namely LDL-cholesterol, between fractions and to evidence the potential health properties of the folate-enriched protein extract obtained from egg yolk.

CHAPTER 5: EFFECT OF SELECTED PRETREATMENTS TO INCREASE THE FOLATE CONTENT OF GRANULE SUSPENSIONS PREPARED FROM HEN EGG YOLK

5.1. CONTEXTUAL TRANSITION

The previous chapter showed that egg fractionation by centrifugation allowed the concentration of folate in granule faction. However, pretreatments could improve folate recovery. Consequently, this Chapter aims to improve concentration of folate in granule fraction by using pretreatment methods naming as increasing ionic strength and power ultrasound. Granule has compact and non-soluble structure and application of pretreatment technique can facilitate re-suspension of granules.

Results obtained in this part of the project will be submitted by:

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This article is in preparation for submission in Journal of Food Engineering. Moreover, the results were presented as a moderate poster presentation in the 17th World Congress of Food Science & Technology (IUFoST 2014) which took place in Montreal, Canada (August 17-21, 2014).

5.2. RÉSUMÉ

Les compositions de granules suite à des prétraitements tels que l'augmentation de la force ionique et l'application d'ultrasons ont été caractérisées. Les résultats du SDS-PAGE ont montré qu'une force ionique élevée aboutit à la solubilisation des protéines modifiant la structure des granules. Pour une concentration de NaCl de 0.15 M, le granule contenait 21 µg folate / g granule extrait. Cependant, pour des concentrations en NaCl supérieures, des traces de folate libéré dans la fraction de plasma ont été détectées. Concernant les ultrasons, les granules remises en suspension ont été prétraitées pendant 5 et 10 min à 95 W. Aucun traitement n'a présente de différence significatif avec le contrôle en termes de composition du granule. Tous les résultats témoignent de la grande stabilité structurelle des granules d'où sa complexité à être dénature. Par conséquent, l'addition de sel augmentant la force ionique démontre l'importance de l'existence de ponts entre les groupes phosphocalciques des protéines granulaires qui maintiennent ce réseau compact et très résistant.

5.3. ABSTRACT

The composition of granules after pre-treatment using increased ionic strength and ultrasound was characterized. The polyacrylamide electrophoresis showed that ionic strength modifications induced the loss of granule structure by solubilisation of proteins. The cholesterol and 5-MTHF content of granules decreased at ionic strength higher than 0.15 M NaCl. At ionic strength 0.15M NaCl granule contained 21 μg folate/g granules extract. However further enhancements was not possible by increasing the salt concentration due to release of folate in plasma fraction. Concerning ultrasound, the re-suspended granules were pretreated during 5 and 10 min at 95 W. The total protein, lipid, cholesterol, fatty acid and 5-MTHF content after ultrasonic pretreated granules was not significantly ($P>0.05$) different between treatments. All results were indicative of the stable structure of granule and that its modification is difficult. Consequently, the application of salt addition and increasing ionic strength indicates the importance of the existence of phosphocalcic bridges between phosphate groups of granular proteins which keeps this network compact and hardly breakable.

5.4. Introduction

Egg yolk is a main ingredient for many food products due to its excellent functional properties. Hen egg yolk is a complex system of an aqueous phase (plasma) and insoluble granules. Native egg yolk can be separated in to its main fractions of granule and plasma by centrifugation techniques. Over the last 40 years, different centrifugal techniques have been studied to separate egg yolk components (Laca et al., 2010; Mc Bee and Cotterill, 1979; Strixner and Kulozik, 2013). Plasma fraction can be solubilized and separated by centrifugation because it is mainly composed of low density lipoproteins with a density of 0.98 g/cm^3 close to density of water (Anton et al., 2003). Granules are major components of yolk containing 70% lipovitellins, 16% phosvitin and 12% low density lipoproteins (LDL) (Causeret et al., 1991). Egg yolk granule mainly composed of proteins and contained low lipid content compare to egg yolk but fractionation procedures could affect the composition of granules (Laca et al., 2014). Folates are a group of B vitamins required for one-carbon metabolism, amino acid biosynthesis, cell division and growth. 5-methyltetrahydrofolate (5-MTHF) is the most biologically active form of B9-vitamin also generically known as folate. Egg yolk has been recognized as an excellent source of folate with high bioavailability where the entire folate is in egg yolk (Strandler, 2012). The folate content in eggs can be enhanced due to the hen's ability to convert synthetic folic acid from fortified feed to natural folate forms (House et al., 2002). Based on recent studies folate-enriched eggs presented as beneficial source of folate with a high bioavailability (Sugiyama et al., 2012).

In previous study, it was demonstrated that fractionation of egg yolk into plasma and granule resulted having granule fractions with higher protein and folate content compare to the native egg yolk (Naderi et al., 2014). Indeed, the fractionated granule at lab-/pilot-scale centrifugation contains 15-20 µg folate/g granule, which corresponds to 5% of the recommended daily allowance of 400 µg folate for adults (DRI, 1998). Whereas no folate was detected in plasma fractions indicates a complete recovery of folate in granules. Consequently, granules with their low-cholesterol content feature (Laca et al., 2010), high protein content and being enriched with 5-MTHF (Naderi et al., 2014) make their application more interesting to be employed as health ingredient.

Granular units consist of a HDL-phosvitin complex. At low ionic strength, granules mainly make non-soluble HDL-phosvitin complexes linked together by phosphocalcic bridges. This is due to the high proportion of phosphoserin amino acids able to bind calcium (Anton, 2013; Castellani et al., 2004; Causeret et al., 1991). The numerous phosphocalcic bridges make the granule structure very compact, poorly hydrated, and weakly accessible to enzymes and lead to an efficient protection against thermal denaturation and heat gelation (Anton, 2013). The structure of granules closely depends on ionic strength and presence of bi-or polyvalent mineral cations. At an ionic strength over 0.3 M NaCl, the phosphocalcic bridges are disrupted because monovalent sodium replaces divalent calcium (Anton et al., 1997). In recent studies, the application of ultrasound was introduced being useful in degradation of high molecular weight proteins and cholesterol in egg yolk (Sun et al., 2011). Granular complex structures form a compact and non-soluble network. The role of possible protein-protein interactions between granule HDL-phosvitin and LDL-apoprotein constituents might be responsible in forming this stable network.

Consequently, the objective of our study was to investigate the effect of increasing ionic strenght or applying ultrasound as pre-treatments to modify egg yolk granule composition and increase its 5-MTHF concentration.

5.5. MATERIALS AND METHODS

5.5.1. Materials and chemicals

Petroleum ether, ethyl ether, chloroform, methanol, acetic acid and hydrochloric acid were purchased from Fisher Scientific (NJ, USA). Sodium chloride, carbon tetrachloride (99.9% purity), (+)-sodium L-ascorbate, and 5-MTHFdisodium salt (90% purity) were obtained from Sigma-Aldrich (MO, USA). Cholesterol (Cholest-5-en-3β-ol with purity of >99%) was purchased from Merck (Darmstadt, Germany). Acetonitrile and ethanol was of HPLC gradient grade and from Sigma-Aldrich (MO, USA); other chemicals were of analytical quality. Water was purified (≤ -0.1 μS cm^{-1}) using a Milli-Q system of Millipore (LA, USA). The standard 5-MTHF was purchased in lyophilized form and was stored at -20ºC until use. The stock solutions of folate for HPLC, 50nM, were prepared in ascorbate buffer (20 g/L sodium ascorbate; 12.1 g/L Trizma base; pH 7.8). Standard reference materials, SRM 1544 and 1946 were purchased from the National Institute of Standards and Technology (NIST).

5.5.2. Preparation of yolk and granules

Fresh white shelled eggs of weight class L (58-63 g) were purchased separately for each batch of experiments from a local supermarket. The eggs were broken manually and the yolks were separated from albumen and chalazae using blotting paper. The vitelline membranes were ruptured by using tweezers and yolk material was collected. The yolk was diluted with Milli-Q water (1:1 v/v) to reduce the viscosity. The egg yolk fractionation was achieved based on the method described by (Naderi et al., 2014), using a tubular bowl centrifuge (CEPA Centrifuge, series LE, Germany) with a maximum centrifugal force of 40,000×g. The diluted fresh egg yolk was pumped into the centrifuge to separate granules from plasma. The sedimented granule fraction was collected and stored at 4ºC for further analysis.

5.5.3. Application of pre-treatments on granule suspensions

Granules were prepared from fresh egg yolks and used as starting material to study the effect of selected pre-treatments on granule composition.

5.5.3.1. Re-suspension with different ionic strength

The granules were diluted (1:2 w/v) in NaCl solutions of increasing ionic strength (0, 0.1, 0.15, 0.25, 0.55M NaCl) and homogenized by Ultra-Turrax T-25 treatment(IKA® Werke Staufen, Germany) for 30s pulses at 6500 rpm.Then samples were centrifuged at 10,000×g for 20 min and the supernatants were separated from precipitated granule and each fraction was collected in separate tubes. Consequently, according to their ionic strength, there were two set of samples: granules (G_{H2O}, $G_{0.1}$, $G_{0.15}$, $G_{0.25}$) and plasma (P_{H2O}, $P_{0.1}$, $P_{0.15}$, $P_{0.25}$). Each set of samples were analysed separately.

5.5.3.1. Ultrasound treatments

The granule fractions separated from yolk were freeze-dried at -70ºC under vacuum by using pilot lyophilizer (VirTis Ultra 50L, SP Scientific, NY, USA). The dry extract of freeze-dried granules was 99.26 ± 0.74 (g/100g). The granules were mixed with ×2 volume of Milli-Q water. The granule solutions were accurately weighted and dissipated with an ultrasonic instrument (Virsonic 475 Cell Disrupter) in a 100 mL sealed flask. The actual power delivered into the system was 95 W at 20% amplitude. An ultrasonic probe with a tip diameter of 7 mm was fitted into the flask and the tip was inserted at the half height of the solution. The ultrasonic process time was set at 0, 5 and 10 min (PULSA^{RTM} on/off for 15s/10s). The temperature of solutions was controlled by immersion the sample flask into an ice-bath. After ultrasound treatment, all samples were centrifuged (10,000×g; 20 min; 23˚C). All the experiments were performed in triplicate. The analyses were performed on each separated fraction. Weights were registered before and after centrifugation.

5.5.4. Chemical analyses

Moisture and total lipid content were determined based on the official AOAC (2005) method. The total protein content of egg yolk and its fractions was determined with a nitrogen gas analyzer system (LECO FP-528, Model 601-500, LECO Co.). The instrument was previously calibrated with ethylenediaminetetraacetic acid (EDTA). Total protein is calculated from the nitrogen content of the yolk material using a conversion factor of 6.25 for all egg samples. Sodium content was determined by inductively coupled plasma (ICP-OES, Optima 4300, Dual View, Perkin-Elmer, Shelton, CT). The wavelength used to determine Na element was 589-592 nm. Each experiment was repeated in triplicate on four different batches of egg samples.

5.5.5. Determination of cholesterol

The cholesterol was extracted from samples using dispersive liquid-liquid microextraction (DLLME) method and analyzed based on the method described by Daneshfar et al. (2009) with some modifications. Approximately 0.1 g of each yolk and granule samples was weighted and added to 10 mL milli-Q water, shaken and centrifuged at 850 ×g for 2 min. The 0.8 mL of solution containing methanol and 35 µL of carbon tetrachloride was added to supernatant (4 mL). The mixture was shaken for 1 min and centrifuged (5311×g; 5 min). The precipitated droplets of fat were air dried at room temperature. The dried samples were re-dissolved in 50 µL of denatured ethanol and centrifuged at 3399 ×g for 2 min. The extracted oil samples were transferred into HPLC vials and injected into the HPLC-UV system. HPLC analysis was performed using an Agilent 1100 HPLC system (Agilent, Palo Alto, CA) equipped with a fluorescence detector (Waters, Milford, MA). The analytical isocratic RP-HPLC separation was performed by using Kinetex™ C18column (75×2.10 mm; 23^{o}C; flow-rate 0.5 mL/min; UV detection wavelength of 210 nm). The mobile phase was made up of acetonitrile and ethanol (50:50, v/v) and a flow rate of 1 ml/ min was used at room temperature. A solution of 1 ppb of cholesterol in 20% ethanol was used for the optimization of DLLME. The software Agilent Chemstation was used to control the HPLC system, and to collect and process the chromatographic data.

5.5.6. Fatty acid analysis

Fatty acids were extracted from the egg yolk according to the methods of Folch et al. (1957). The fatty acid composition of the samples was determined using standard gas chromatographic techniques of the fatty acid methyl esters (AOAC, 1990), using C17:1 methyl ester as an internal standard.

5.5.7. Determination of folate content

Folate was extracted from samples according to the method of House et al. (2002) with some modifications. The yolk and granule samples were weighted (0.5 g) into falcon tubes (15 mL). The extraction buffer (20 g/L sodium ascorbate; 12.1 g/L Trizma base; pH 7.8) was added to samples, vortexed, and placed in a boiling water bath for 60 min. Then, tubes were rapidly centrifuged (3000×g; 35 min; 4ºC) and supernatants were decanted and stored at 4ºC. The extraction procedures were repeated one more time and the final volume of collected supernatants were brought to 25 mL. The samples were passed through 0.2 µm nylon membrane filter and transferred into amber vials for further analysis. The folate (5-MTHF) was detected and quantified using RP-HPLC analysis using an Agilent 1260 HPLC system (Agilent Technologies Inc., Palo Alto, CA) equipped with a 474 fluorescence detector from Waters (Milford, MA). Folate was quantified using fluorescence detection at excitation 290 nm and emission 360 nm.

5.5.8. SDS–PAGE protein profiles

Protein solutions (1%) were prepared and hydrated at 4ºC overnight. The protein samples were further diluted to reach a protein in each gel lane of about 25 µg. Samples were diluted (1:1 v/v) in a dissociation buffer consisting of a 0.5 M Tris–HCl pH 6.8, 0.5% bromophenol blue, 35% glycerol, 5% β-mercaptoethanol, 10% (w/v) SDS solution, and heated in a boiling water for 5 min. Electrophoresis were run on precast 4-20% gradient polyacrylamide gels (Bio-Rad, CA) with a migration buffer consisting of a 0.02 M Tris

(hydroxymethyl) aminomethane, glycine 5 M and SDS (w/v) 0.1% solution. The proteins were stained using Coomassie blue method which was modified by addition of aluminum nitrate for detection of phosphoproteins (Hegenauer et al., 1977). After destaining in a solution containing acetic acid/ methanol/water (10:40:50 v/v/v), the gels were scanned with a ChemiDOC™ XRS (Bio-Rad, CA) system. Molecular weights were estimated by a molecular weight standard (Precision Plus Protein™ Standards, Bio-Rad Laboratories Ltd, CA).

5.5.9. Statistical analysis

All analysis was repeated on four different batches of granule samples. Data were expressed as mean ± standard deviations of at least triplicate determinations unless specifically mentioned. A multiple analysis of variance (MANOVA) model was used to test for significant differences in granule compositions among treatments. Computations were done using the general linear model procedure. Statistical calculation was investigated using the statistical package Minitab Statistical Software release 16.2.3 for Windows (Minitab®, State College, PA) for one-way analyses of variance. Confidence intervals were set at 95% ($P<0.05$).

5.6. Results and discussion

The composition of the granules separated from egg yolk by using the tubular bowl centrifuge presented in Table 5.1. The granules represented 69.40 (± 0.10) g/100 g of proteins on dry basis which is nearly 60% of the protein content of the yolk. The 5-MTHF concentration retained in granule fraction was 2107.3 (±43.1) µg/100g which is in accordance with previous study (Naderi et al., 2014). Granules contain low concentration of lipid since the ratio of lipid/protein was 0.3 compare to 2.1 in yolk. Cholesterol content was limited in granule since the ratio of cholesterol in granule to yolk was 0.3.

Table 5.1. Composition of granule fraction separated from yolk on dry basis. Average of twelve replications from four batches.

	Yolk	Granule
Protein (g/100g)	30.6 ± 0.2	69.4 ± 0.1
Lipid (g/100g)	65.4 ± 4.4	25.3 ± 1.1
5-MTHF (μg/100g)	460.1 ± 56.6	2107.3 ± 43.1
Cholesterol (mg/100g)	89.2 ± 15.6	28.5 ± 12.7
Lipid/protein	2.1	0.3

5.6.1. Effect of ionic strenght

5.6.1.1. Protein and folate recovery

The recovery rate of granules after solubilization and centrifugation was between 95-100% (Table 5.2) for each treatment. The recovery rate of protein and 5-MTHF from granules was calculated as 88-100% and 74-187%, respectively. Granules in 0.55 M NaCl solution produced a higher recovery rate for 5-MTHF. Complete disruption of granules were occurred in saline solution with ionic strength 0.55M NaCl, where granule particles dispersed and formed yellow-translucent suspensions. At this condition, further separation of granules were not possible by centrifugation.

Table 5.2. Recovery of granules and major components: protein and folate (n=4)

Treatment	%Yield of granules after treatments	%Yield of protein	%Yield of 5-MTHF
Water	95.1 ± 3.7	99.9 ± 3.5	84.6 ± 19.9
0.1M NaCl	101.6 ± 2.3	92.1 ± 5.1	90.5 ± 30.4
0.15M NaCl	100 ± 4.4	100.6 ± 6.8	87.4 ± 27.1
0.25M NaCl	100.9 ± 2.1	96 ± 4.8	74.4 ± 30.7
0.55M NaCl	101.2 ± 2.9	88.1 ± 3.2	124.3 ± 3.6

5.6.1.2. Granule and plasma composition

Table 5.3 compares composition of granules and plasma between each treatment as a function of ionic strength. The protein content of the granules at low ionic strength of 0.1 M NaCl was significantly different from protein content of granule at higher ionic strength (IS>0.1 M NaCl). However, at IS 0.15, 0.25 M NaCl the protein concentration of granule was not significantly different. At low ionic strength, granules mainly form non soluble lipovitelin (HDL) and phosvitin complexes which can be precipitated by centrifugation (Causeret et al., 1991). It was believed that in granular structure, HDL and phosvitins are bounded through phosphocalcic bridges between their seryl residues and they form nonsoluble complexes (Le Denmat et al., 1999). The phosphoserine residues of Ca^{2+} are negatively charged and by addition of the salt the divalent Ca^{2+} are substituted by monovalent Na^{+} and subsequently phosphocalcic bridges are broken, and phosvitin and HDL become soluble (Anton et al., 2000b).

The granule re-suspended in water (G_0) had significantly ($P<0.05$) lower lipid content comapre to control granule. This is due to the significant lower dry material in granul after re-suspension and centrifugation. The lipid concentrationof granule was significantly lower (12.5±3.1 g/100g on dry basis) at ionic strength 0.25M NaCl. As stated before, at higher ionic strength (>0.15M), hydrophilic phosvitin become soluble and HDLs behave like soluble proteins. Thereby, as the effect of 0.25M NaCl the precipitate granules had 50% less lipid in their structure which was separated through plasma after centrifugation.

As mentioned previously, the presence of a large amount of phosphocalcic bridges in the granule structure makes its very compact and poorly hydrated. Lipid content of granules is associated with HDLs which is principally composed of phospholipids and neutral lipid. The X-ray crystallography of HDL demonstrated that this protein contains a funnel-shaped cavity formed primarily by two β-sheets and filled with hydrophobic lipid. The charged hydrophilic phospholipid head groups form the aqueous interface at the mouth of the funnel and interact with the ring of basic and polar side chains of protein (Anderson et al., 1998). The phospholipid present in the form of a condensed state such as monolayer (Banaszak et

al., 1991) that their polar portions are exposed on the lipoprotein surface, thus making the particle soluble in aqueous solution.

Table 5.3. Composition of granules and plasma as a function of ionic strength variation (NaCl concentration at 4 points)[1]

	G_C [2]	G_0	$G_{0.1}$	$G_{0.15}$	$G_{0.25}$	P_0	$P_{0.1}$	$P_{0.15}$	$P_{0.25}$
Protein (g/100g dry basis)	69.5 ± 2.2^{bc}	73.1 ± 2.5^{c}	65.0 ± 5.2^{b}	74.6 ± 7.8^{c}	73.6 ± 3.4^{c}	0.7 ± 0.6^{b}	0.3 ± 0.1^{a}	0.2 ± 0.0^{a}	0.2 ± 0.1^{a}
Lipid (g/100g dry basis)	21.8 ± 1.9^{b}	27.6 ± 0.9^{d}	24.6 ± 2.0^{c}	22.5 ± 2.5^{bc}	12.5 ± 3.1^{a}	0.8 ± 0.7^{a}	0.7 ± 0.5^{a}	0.5 ± 0.2^{a}	0.7 ± 0.3^{a}
5-MTHF (µg/100g dry basis)	1964.9 ± 253.9^{a}	2087.4 ± 448.7^{a}	2075.0 ± 261.2^{a}	2128.2 ± 290.2^{a}	1883.3 ± 407.8^{a}	ND[3]	ND	1.5 ± 0.2^{a}	2.3 ± 0.8^{a}
Cholesterol (g/100g dry basis)	28.5 ± 12.7^{a}	125.0 ± 67.1^{c}	93.3 ± 50.9^{bc}	70.4 ± 21.7^{b}	26.3 ± 11.0^{a}	3.9 ± 3.3^{a}	4.3 ± 2.8^{a}	5.4 ± 3.5^{a}	6.3 ± 4.6^{a}
Dry matter (g/100g)	49.9 ± 0.7^{a}	44.0 ± 2.2^{b}	42.8 ± 1.7^{b}	43.1 ± 2.6^{b}	42.9 ± 0.7^{b}	0.4 ± 0.0^{a}	1.4 ± 0.1^{b}	2.2 ± 0.1^{c}	3.9 ± 0.6^{d}
Ash (g/100g)	3.2 ± 0.3^{b}	3.3 ± 1.7^{b}	2.5 ± 1.0^{b}	2.8 ± 0.3^{b}	3.3 ± 0.3^{b}	0.02 ± 0.0^{a}	0.5 ± 0.1^{b}	0.7 ± 0.04^{c}	0.99 ± 0.2^{d}
Na (g/L)	0.1 ± 0.1^{a}	0.2 ± 0.2^{a}	0.5 ± 0.2^{ab}	1.2 ± 0.6^{b}	2.4 ± 1.2^{c}	0.13 ± 0.1^{a}	1.7 ± 0.2^{b}	2.7 ± 0.3^{c}	4.1 ± 0.9^{d}

[1] Values are mean ± SD. Comparison analysis was done among plasma and granule samples separately.

[2] GC=Granule control; Granule in native state fractionated from yolk and used as control in all experiments.

[3] ND= not detected.

[a-c] Values with common superscripts in each row are not significantly different (P>0.05).

No significant different (P>0.05) were observed for 5-MTHF concentration of granule whatever ionic strength variation. At lower ionic strength (0.1M NaCl) 5-MTHF was not separated from granular structure along with plasma after centrifugation. However, increasing ionic strength to 0.15M and 0.25M liberated low concentration of 5-MTHF in plasma fraction. Marginal changes in 5-MTHF content as the effect of ionic strength was similar to protein values, since the ratio of protein/folate remained always 0.03.

The values obtained for cholesterol as the effect of ionic stregth variation was higher (P<0.05) compared to control granule. At 0.25M NaCl, cholesterol content of granule was 26.3 (± 11.0) g/100g on dry basis which was lower than control granule (28.5 ± 12.7 g/100g dry basis). The total cholesterol content of the control granule (P < 0.05) was lower than the re-suspended granule at lower ionic strength (IS= 0, 0.1, 0.15 M NaCl). The total cholesterol is representative of cholesterol esters and free cholesterol and differences in the cholesterol content after re-suspension of granule at low ionic strength might be due to the location of cholesterol esters and free cholesterol in granule. Cholesterol esters may be less available for extraction, as they are located in the core of the low density lipoproteins surrounded by an amphiphilic coating of protein, phospholipid, and free cholesterol.

By increasing the ionic strength the dry matter of granules was not significantly (p<0.05) different. Total ash was not different (P>0.05) among treatment because sodium allowed the movement of bivalent cations which were separated in plasma phase. Sodium concentration was measured in control granule as 0.1 (± 0.1) g/L as indicator to control the salt concentration after treatments. Consequently, the increase of ionic strength increase sodium concentration in granules and plasma

5.6.1.3. Fatty acids profile of granules and plasma:

Fatty acid composition of the granules and plasma fraction after increasing ionic strength and separation was presented in Table 5.4. Fatty acid composition of native egg yolk were also analyzed in order to be used as reference. The presence of twelve fatty acids was determined in egg yolk. Oleic acid (C18:1), palmitic acid (C16:0), linoleic acid (C18:2) and stearic acid (C18:0) were respectively the main fatty acids measured in the egg yolk and

granule samples. The fatty acid results for egg yolk were similar to the results reported by Wang et al (2000b). However, the marginal different in reported values might be related to fatty acid dietary sources in hen's diet. Researche shows that yolk lipid composition can be influenced by fatty acid dietary sources particularly for n-3 poluunsaturated fatty acids (PUFA) (Souza et al., 2008, Goldberg et al., 2012). Fatty acid profile of granules were similar to yolk except eicosapentaenoic acid (EPA, $C20:5_{n-3}$) which was not detected in granule samples. By fractionation of egg yolk, only half of fatty acids ($P<0.05$) separated in granular fraction.

Changing the ionic strength in granule fraction showed significant difference ($P<0.05$) in the fatty acid composition of granules. However, at lower salt concentrations (0.1, 0.15M NaCl), the values obtained for granule was slightly higher than control granule. This can be explained by the hydrophilic interaction between the charged hydrophilic phospholipid headgroups and water molecules in aqueous solution which improved recovery of fatty acids. Total saturated fatty acids (SFA), PUFA, monounsaturated fatty acids (MUFA), unsaturated to saturated fatty acid ratio (UFA:SFA), and PUFA:SFA ratio were calculated for yolk, granule and granules disrupted after increasing ionic strength (Table 5.4).

Table 5.4. Fatty acid composition of granules and plasma as the function of ionic strength variation (NaCl concentration at four points)[1]

				Granules after the effect of ionic strength variation				Plasma after the effect of ionic strength variation		
Fatty acids	Yolk	G_C [2]	G_0	$G_{0.1}$	$G_{0.15}$	$G_{0.25}$	P_0	$P_{0.1}$	$P_{0.15}$	$P_{0.25}$
C14:0	1.47 ± 0.01^{a}	0.52 ± 0.03^{b}	0.63 ± 0.02^{bc}	0.58 ± 0.00^{c}	0.57 ± 0.04^{cd}	0.46 ± 0.01^{de}	0.003 ± 0.00^{c}	0.02 ± 0.00^{a}	0.01 ± 0.00^{b}	0.02 ± 0.00^{a}
C16:0	103.68 ± 0.52^{a}	46.03 ± 2.30^{b}	50.96 ± 2.21^{bc}	47.53 ± 0.65^{bc}	46.35 ± 2.91^{c}	39.28 ± 0.59^{d}	0.28 ± 0.00^{c}	0.67 ± 0.05^{b}	0.73 ± 0.02^{b}	1.08 ± 0.13^{a}
C18:0	36.18 ± 0.18^{a}	17.92 ± 0.90^{b}	18.85 ± 0.82^{b}	18.75 ± 0.26^{b}	18.32 ± 1.15^{bc}	16.15 ± 0.24^{c}	0.13 ± 0.00^{c}	0.24 ± 0.01^{b}	0.26 ± 0.01^{b}	0.37 ± 0.04^{a}
C16:1	11.25 ± 0.06^{a}	4.56 ± 0.23^{b}	5.53 ± 0.24^{c}	4.70 ± 0.06^{c}	4.40 ± 0.28^{c}	3.56 ± 0.05^{d}	0.02 ± 0.00^{c}	0.09 ± 0.01^{b}	0.08 ± 0.00^{b}	0.13 ± 0.01^{a}
C18:1	169.24 ± 0.85^{a}	64.30 ± 3.22^{b}	71.81 ± 3.12^{bc}	70.62 ± 0.97^{bc}	69.88 ± 4.39^{cd}	56.55 ± 0.85^{de}	0.26 ± 0.18^{c}	1.09 ± 7.95^{b}	1.18 ± 3.03^{b}	1.64 ± 19.45^{a}
C18:2	56.61 ± 0.28^{a}	24.60 ± 1.23^{b}	27.15 ± 1.18^{b}	26.60 ± 0.36^{b}	26.07 ± 1.64^{b}	21.91 ± 0.33^{c}	0.10 ± 0.00^{c}	0.35 ± 0.02^{b}	0.40 ± 0.01^{b}	0.60 ± 0.07^{a}
GLA	0.47 ± 0.00^{a}	0.26 ± 0.01^{b}	0.28 ± 0.01^{b}	0.23 ± 0.01^{c}	0.23 ± 0.01^{c}	0.20 ± 0.00^{d}	0.00± 0.00^{c}	0.05 ± 0.00^{b}	0.04 ± 0.00^{b}	0.06 ± 0.01^{c}
ALA	3.09 ± 0.02^{a}	0.43 ± 0.02^{b}	0.52 ± 0.02^{b}	1.24 ± 0.02^{c}	1.24 ± 0.08^{d}	0.97 ± 0.01^{e}	0.00± 0.00^{d}	0.03 ± 0.00^{c}	0.02 ± 0.00^{b}	0.01± 0.00^{a}
C20:4	7.92 ± 0.04^{a}	6.12 ± 0.31^{b}	6.03 ± 0.26^{b}	5.62 ± 0.08^{bc}	5.43 ± 0.34^{c}	5.24 ± 0.08^{c}	0.03 ± 0.00^{c}	0.06 ± 0.00^{b}	0.06 ± 0.00^{b}	0.09 ± 0.01^{a}
EPA	0.12 ± 0.00	ND[3]	ND	ND	ND	ND	ND	ND	ND	ND
DPA	0.42 ± 0.00^{a}	0.16 ± 0.01^{b}	0.15 ± 0.01^{b}	0.26 ± 0.00^{c}	0.26 ± 0.01^{d}	0.23 ± 0.00^{e}	ND	ND	ND	ND
DHA	4.96 ± 0.02^{a}	1.81 ± 0.09^{b}	1.86 ± 0.08^{b}	3.60 ± 0.05^{b}	3.49 ± .22^{c}	3.36 ± 0.05^{d}	ND	ND	ND	ND
SFA	141.34 ± 0.71^{a}	64.48 ± 3.23^{b}	70.44 ± 3.06^{b}	66.86 ± 0.92^{b}	65.25 ± 4.10^{b}	55.90 ± 0.84^{c}	0.41 ± 0.00^{c}	0.92 ± 0.07^{b}	1.01 ± 0.02^{b}	1.46 ± 0.17^{a}
MUFA	237.10 ± 1.19^{a}	93.46 ± 4.68^{c}	104.50 ± 4.54^{b}	101.92 ± 1.40^{bc}	100.35 ± 6.30^{bc}	82.03 ± 1.24^{d}	0.37 ± 0.00^{c}	1.53 ± 0.11^{b}	1.65 ± 0.04^{b}	2.34 ± 0.28^{a}
PUFA	16.98 ± 0.08^{a}	8.78 ± 0.44^{c}	8.84 ± 0.38^{c}	10.96 ± 0.15^{b}	10.65 ± 0.67^{b}	10.00 ± 0.15^{b}	0.03 ± 0.00^{c}	0.21 ± 0.01^{b}	0.16 ± 0.00^{a}	0.19 ± 0.02^{ab}
UFA: SFA*	1.80	1.58	1.61	1.69	1.70	1.65	0.99	1.88	1.80	1.72
PUFA: SFA*	0.12	0.14	0.12	0.16	0.16	0.18	0.08	0.23	0.16	0.13
Total omega-6	65.00 ± 0.33^{a}	30.98 ± 1.55^{b}	33.46 ± 1.45^{b}	32.45 ± 0.44^{b}	31.73 ± 1.99^{b}	27.35 ± 0.41^{c}	-[4]	-	-	-
Total omega-3	8.59 ± 0.04^{a}	2.40 ± 0.12^{b}	2.53 ± 0.11^{b}	5.11 ± 0.07^{c}	4.99 ± .31^{cd}	4.55 ± 0.07^{d}	-	-	-	-
n-6: n-3	7.57	12.2	13.21	6.35	6.35	6.00	-	-	-	-

[1] Values are mean ± SD (mg/g yolk or granule on dry basis).
[2] GC = granule control; Granule in native state fractionated from yolk and used as control in all experiments.
[3]ND=not detected.
[4] Negligible amounts. Not calculated.
[a-g] Values with common superscripts in each rows are not significantly different (P>0.05).
*: Means significantly different.

Total SFA content of granules (64.48 ± 3.23) was lower than the one measured for egg yolk (141.34 ± 0.71) with a ratio of 1:2. Lower SFA content was calculated for granule at ionic strength of 0.25M NaCl which was 55.90 (± 0.84) mg/g granules on dry basis compare to control granule (64.48 ± 3.23 mg/g granule on dry basis).

Ionic strength significantly influenced total MUFA of granules at 0.25M NaCl but has no effect on PUFA content. The ratio of PUFA: SFA and UFA: SFA in granules was increased by increasing the ionic strength indicating the effect of salt in separating saturated fatty acids from granular fraction.

Total omega-6 and omega-3 fatty acids were calculated for yolk and granule fractions. The control granule has two times less omega-6 (30.98 ± 1.55 mg/g) fatty acids compared to native egg yolk (65.00 ± 0.33 mg/g). The calculated total omega-6 fatty acids for granule fraction were lower than total omega-3 fatty acids. The omega-6 (n-6) to omega-3 (n-3) ratio was calculated for the granule fractions as the effect of ionic strength variation. This ratio was 12.1 for control granule however this ratio decreased by half while increasing the ionic strength. From the nutritional stand point, lower values for this ratio are more desirable. According to Simopoulos (2002), lower ratio of omega-6 to omega-3 fatty acids is more desirable in diets. Granules are composed of 70% lipovitellins or HDLs. However, their structure is different from that of lipoproteins. Unlike LDLs, HDLs do not have a core of neutral lipids surrounded by a layer of phospholipids and apoproteins.

HDLs mainly composed of proteins and their lipid content is composed of phospholipids that are bound to proteins as ligands mainly in the N-terminal region, by hydrophobic interactions (Banaszak and Seelig, 1982). The structure of HDL is held together by five disulphide bridges and comprises a hydrophobic cavity. It was shown that most of HDL lipids are bound in this hydrophobic cavity (Timmins et al., 1992). Granule does not have a homogeneous composition (Strixner and Kulozik, 2013) and its different subfractions can employ different lipid content. The main fatty acids in plasma were palmitic acid (C16:0)>Oleic acid (C18:1)>stearic acid (C18:0)>linoleic acid (C18:2).

The results of present experiment indicate that an increase of ionic strength modified the fatty acid profile of granule. Higher concentration of NaCl had more effect on separation of

C18:1, C18:2, C16:0 and C18:0, respectively. Moreover, with increasing ionic strength, higher values were also calculated for total SFA, PUFA and UFA in plasma fraction. Based on the obsevations, ionic strengh of 0.15 M NaCl significantly ($P<0.05$) effect the fatty acid composition of disrupted granules.

5.6.1.4. Protein distribution (SDS-PAGE)

The protein profile of granule fraction and separated plasma from resuspended granules were analysed by SDS-PAGE. The electrophoretic pattern of granules versus plasma proteins was obtained in a highly reproducible way in our analysis. The Figure 5.1-lane a shows protein composition of control granule which was from direct fractionation of egg yolk. The total 6 bands with molecular weights of 110, 77, 59, 40, 45 and 35 kDa was assigned to the proteins of control granule. The band 1 and 2 with molecular weight of 110 kDa correspond to apovitellin 3+4, band 3 to apovitellin 5+6 (78kDa), band 4 to apovitelin 7 (59 kDa) and band 6 (35 kDa) to vitellogenin-3 (theoretical Mw of 38 kDa). In the current study, band 5 was assigned to phosvitin with molecular weight of 45 kDa by comparing with commercial phosvitin.

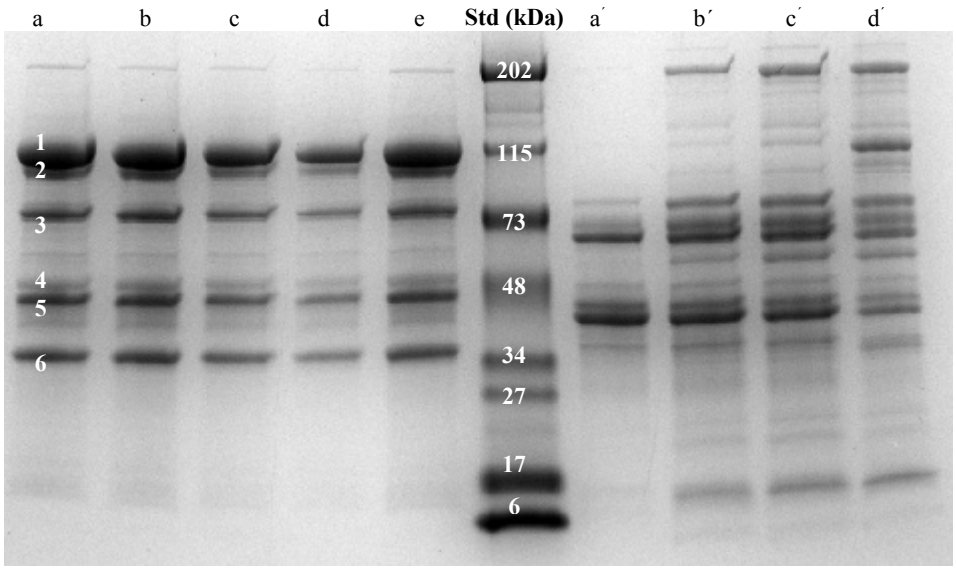

Figure 5.1. Electrophoretic profile of total granule proteins after changing ionic strength at 0, 0.1, 0.15, 0.25, 0.55 M NaCl (a-e) and soluble proteins in plasma fraction separated from granules (a´ - d´). Std=MW standard.

Lanes “b-e” show the protein composition of granules after ionic strength variation treatment. Addition of NaCl to a granule solution resulted in solubilization of the granular proteins. At ionic strength of 0.1, 0.15, 0.25M NaCl, granule particles forme a milky solution and by further centrifugation the separation of granules from plasma solutions was posible. At 0.25M NaCl, the granular structure were partly disrupted and by further centrifugation two distict yellow fraction were separated. Protein composition of granules in each treatment was similar to the control granule and the four major apovitellins and phosvitin are clearly visible. The intensity of protein bands decreased across granule profiles by increasing the ionic strength. At 0.55M NaCl, the granular structure completely disrupted and formed a homogen and translucent solution. The protein profiles at this ionic strength (lane e) was compared with the granules in lane a. It was noticed that composition of proteins at two lanes consist of six major bands that can be clearly identified but with different intensities. This indicates that granules has a compact structure in which lipovitellin and phosvitin forms strong complexes thereby in lane a the bands had higher intensity. In lane “e” where lipovitellin-phosvitin completely dissociated the protein bands migrated at the same molecular weight but they were detectable with the same intensity.

By comparing protein profile of granules (a, b, c, d) with plasma fraction (a′, b′, c′, d′) separated from granule, we noticed that some proteins were solubilized and separated in plasma by changing the salt concentration. By increasing the ionic strength (0.1, 0.15, 0.25M NaCl), the presence of a protein band at molecular weight of 210 kDa became more visible in plasma fraction (b′, c′, d′) separated from granule. This band can be assigned to vitellogenin II (lipovitellin-2) with theoretical molecular weight of 204 kDa. In lane a′, only water soluble proteins were appeared between molecular weights of 74 and 31 kDa. The bands with apparent molecular weights of 44 and 35 kDa might correspond to phosvitin which appears with aluminum nitrate in our staining solution. These results confirmed previous reports that phosvitin has several components (α1, α2, and β-phosvitin) and that the major component is β-phosvitin (45 kDa) (Castellani et al., 2003). It was reported that phosvitin composed of several polypeptides that interacts or polymerizes in aqueous solution to form larger molecular weight aggregates. And they can be dissociated in several components with MW ranging between 18.5-60 kDa (Abe et al., 1982). Band 1, 2 (lane a; apovitellin 3+4) at MW of 110 kDa may be a heterodimer since the intensity of this band

decreased in lanes b-e. By increasing the NaCl concentration this band degraded to several bands and migrated at lower molecular weight which were appeared in lanes a´- d´. The HDLs in granule present in the form of a dimer of 2 monomers with a molecular weight of about 200 kDa each. Each monomer of HDL is a very structured globular protein (Anderson et al., 1998). HDLs precipitated in water and it was reported that they behave like soluble proteins after separation of water souble phosvitin when ionic strength is higher than 0.3 M NaCl (Anton, 2007b). Thereby, the presence of Vitellogenin II became more visible in plasma fraction by increasing ionic strength. It was presented that each monomer of HDL is composed of about 5 main apoproteins, with molecular weights ranging from 35 to 110 kDa. The appearanc of several protein bands in the plasma fraction after the effect of ionic strength suggest that HDL apoproteins may be organized as heterodimers which consist of different molecular weight polypeptides which were dissociated under the conditions used in this study.

5.6.1. Effect of ultrasound treatment on granule and plasma

5.6.1.1. Protein and folate recovery

The granules were resuspended in distilled water and pre-treated by using ultrasonic at different time points. The overall mass balance and component mass balance was calculated for granules disrupted by ultrasound and the final yields are presented in Table 5.5. It was calculated that more than 98% of granules were recovered after centrifugation. Moreover, after ultrasonic treatment, high recovery of protein and 5-MTHF was obtained for granular fraction.

Table 5.5. Recovery of granules and major components: protein and folate

Ultrasonic time	%Yield of granules after treatments	%Yield of protein	%Yield of 5-MTHF
0 min	102.59 ± 0.00	96.24 ± 3.40	101.22 ± 0.00
5 min	98.15 ± 1.73	99.92 ± 1.72	99.27 ± 8.04
10 min	101.29 ± 3.34	106.50 ± 1.50	105.31 ± 11.15

5.6.1.2. Granule and plasma composition

The composition of granules after pre-treatment with ultrasound in three different time point was analyzed and presented in Table 5.6. By increasing the ultrasonic processing time, no significant (P>0.05) effect on total protein and lipid content of granules and plasma was observed. However, the 5-MTHF content of granule increased from 2012.9 (±52.9) µg/100g at time 0 to 2114.9 (±115.8) µg/100g at 10 min. Nevertheless, 5-MTHF content of granule was not significantly (P=0.10) different whatever ultrasonic treatments applied. Concerning plasma fraction, no folate was detected after ultrasonic treatment and centrifugation. Ultrasonic pre-treatment was used to facilitate dispersion of non-soluble granules in water and interestingly the folate content of granules did not changed across the treatments. The application of ultrasonic assisted extraction in food processing technology has been considered interesting for enhancing extraction of components from animal materials and it is adaptable for both laboratory and industrial scale (Vilkhu et al., 2008). The cholesterol content of granules increased across the ultrasonic treatments. Compare to cholesterol content of control granule 11.36 (±0.95) g/100g, higher values were measured for ultrasound treated granules. Minimal amount of cholesterol was separated in plasma fraction after centrifugation but the ultrasonic treatments had no significant (P=0.23) effect on overall cholesterol concentration of plasma fraction.

Based on our results, ultrasound might slightly increase the concentration of 5-MTHF in final granule extract but it conversely increase the measurable cholesterol. The ultrasound had no significant effect on total protein content, however higher concentration of protein was measured for granule by increasing the ultrasonic processing time. In our study, the application of ultrasound provided granules enriched with 21μg/g ganules similar to the numbers calculated for resuspended granule in saline solution with 0.15M NaCl concentration. The dispersion of compact granular structure was faciliated by application of ultrasound power which was directly reflected in measurable parameters.

Table 5.6. Effect of ultrasonic pre-treatment on composition of granule and separated-plasma from granules after treatments[1]

Composition[2]	Granule Control	Granules			Plasma		
		Treatment			Treatment		
		0 min	5 min	10 min	0 min	5 min	10 min
Protein (g/100g)	70.79 ± 0.4[a]	65.96 ± 1.5[a]	71.99 ± 2.2[a]	74.26 ± 2.9[a]	0.97 ± 0.1[a]	0.77 ± 0.1[a]	0.79 ± 0.2[a]
Lipid (g/100g)	24.84 ± 0.4[a]	22.67 ± 0.1[a]	24.79 ± 1.19[a]	23.05 ± 0.5[a]	0.41 ± 0.1[a]	0.74 ± 0.1	0.78 ± 0.2[a]
5-MTHF (μg/100g)	2001.5 ± 27.9[a]	2012.9 ± 52.9[a]	1958.0 ± 297.5[a]	2114.9 ± 115.8[a]	ND[3]	ND	ND
Cholesterol (g/100g)	11.36 ± 0.9[a]	52.74 ± 2.8[b]	43.87 ± 5.15[b]	61.18 ± 3.96[b]	2.28 ± 0.0[a]	2.44 ± 0.7[a]	1.92 ± 0.6[a]

[1] Values are mean ± SD.
[2] Compositions are on dry basis.
[3] ND= Not Detected.
Values across treatments for granules and plasma separately in each row are not significantly different (P>0.05).

5.6.1.3. Fatty acids profile of granules and plasma

The faty acid composition of granules and separated plasma after pre-treatment with ultrasonic in three different time point was analysed and presented in Table 5.7. The main fatty acids measured (on dry basis) for control granules were C18:1 (50.87), C16:0 (38.11), C18:2 (19.58), and C18:0 (14.25). The fatty acid composition of ultrasonic treated granules was increased slightly by increasing the pulsing time period to 10 min. However, no significant ($P>0.05$) differences were observed in fatty acid composition of granule whatever ultrasonic treatment applied. Marginal changes in fatty acid composition of plasma were negligible ($P>0.05$). The total SFA, MUFA and UFA was calculated for granule samples and it was observed that total MUFA was higher than total SFA. For control granule, the ratio of UFA:SFA and PUFA:SFA was respectively calculated as 1.53 and 0.12. This ratio remained almost unchanged for ultrasonic treated granules at different time points. The ultrasonic treatment might be an interesting choice for dissipation of granules but more investigation is required in order to monitor the composition and structural changes of granule under such condition.

Table 5.7. Fatty acid composition of granule and plasma separated from granule as a function of ultrasonic treatment (ultrasonic process time at three points)[1]

Fatty acids	Granule Control	Granules Treatment			Plasma Treatment		
		0 min	5 min	10 min	0 min	5 min	10 min
C14:0	0.47 ± 0.1[a]	0.51 ± 0.0[a]	0.52 ± 0.0[a]	0.53 ± 0.0[a]	0.02 ± 0.0[a]	0.01 ± 0.0[b]	0.01 ± 0. 0[b]
C16:0	38.11 ± 7.5[a]	42.10 ± 3.1[a]	44.39 ± 1.5[a]	44.36 ± 1.0[a]	0.83 ± 0.3[a]	0.47 ± 0.0[a]	0.57 ± 0.1[a]
C18:0	14.25 ± 2.9[a]	15.79 ± 1.0[a]	16.83 ± 0.6[a]	16.68 ± 0.4[a]	0.26 ± 0.1[a]	0.16 ± 0.0[a]	0.20 ± 0.0[a]
C16:1	4.11 ± 1.3[a]	4.37 ± 0.8[a]	4.59 ± 0.6[a]	4.75 ± 0.3[a]	0.10 ± 0.0[a]	0.05 ± 0.0[a]	0.06 ± 0.0[a]
C18:1	50.87 ± 11.9[a]	57.27 ± 5.0[a]	60.11 ± 2.2[a]	59.99 ± 1.3[a]	1.24 ± 0.5[a]	0.69 ± 0.0[a]	0.80 ± 0.2[a]
C18:2	19.58 ± 4.2[a]	22.39 ± 1.5[a]	23.40 ± 0.8[a]	22.76 ± 0.4[a]	0.38 ± 0.1[a]	0.23 ± 0.0[a]	0.26 ± 0.1[a]
GLA	0.20 ± 0.0[a]	0.23 ± 0.0[a]	0.24 ± 0.0[a]	0.23 ± 0.0[a]	0.004 ± 0.0[a]	0.001 ± 0.0[a]	0.001 ± 0.0[a]
ALA	0.36 ± 0.1[a]	0.40 ± 0.0[a]	0.41 ± 0.0[a]	0.39 ± 0.0[a]	0.01 ± 0.0[a]	0.01 ± 0.0[a]	0.01 ± 0.0[a]
C20:4	4.47 ± 1.0[a]	4.95 ± 0.2[a]	5.35 ± 0.2[a]	5.25 ± 0.1[a]	0.02 ± 0.0[a]	0.02 ± 0.0[a]	0.03 ± 0.0[a]
EPA	ND	ND	ND	ND	ND	ND	ND
DPA	0.12 ± 0.0[a]	0.13 ± 0.0[a]	0.14 ± 0.0[a]	0.14 ± 0.0[a]	ND	ND	ND
DHA	1.26 ± 0.3[a]	1.50 ± 0.1[a]	1.61 ± 0.1[a]	1.49 ± 0.0[a]	ND	ND	ND
SFA	52.83 ± 10.5[a]	58.39 ± 4.2[a]	61.75 ± 2.2[a]	61.57 ± 1.5[a]	1.11 ± 0.4[a]	0.63 ± 0.0[a]	0.78 ± 0.2[a]
MUFA	74.57 ± 17.4[a]	84.04 ± 7.3[a]	88.10 ± 3.6[a]	87.50 ± 1.5[a]	1.72 ± 0.7[a]	0.97 ± 0.0[a]	1.13 ± 0.3[a]
PUFA	6.41 ± 1.4[a]	7.23 ± 0.3[a]	7.77 ± 0.3[a]	7.50 ± 0.2[a]	0.03 ± 0.0[a]	0.03 ± 0.0[a]	0.05 ± 0.0[a]
UFA:SFA*	1.53 ± 0.0[a]	1.56 ± 0.0[a]	1.55 ± 0.0[a]	1.54 ± 0.0[a]	1.57 ± 0.0[ab]	1.59 ± 0.0[a]	1.49 ± 0.0[b]
PUFA:SFA*	0.12 ± 0.0[a]	0.12 ± 0.0[a]	0.12 ± 0.0[a]	0.12 ± 0.0[a]	0.03 ± 0.0[c]	0.05 ± 0.0[b]	0.06 ± 0.0[a]

[1] Values are mean ± SD (mg/g granule and plasma on dry basis).

[2] ND=not detected.

[a-d] Values with common superscripts in each rows are not significantly different (P>0.05). Comparison analysis performed among treatments for granules and plasma separately.

*: Means significantly different (P<0.05) among treatments for plasma fraction.

5.6.1.4. Protein distribution (SDS-PAGE)

Further investigation was done to monitor the effect of ultrasound treatment on the protein profile of granules. The electrophoresis pattern of the granule fraction (Figure 5.2) showed the presence of six bands in control granule (G_{ctr}) which were the characteristic of granule fraction. The intensity of the protein bands among treatments did not changed for granule fraction but several bands were appeared in plasma separated from granules. The number of 6 bands were detected in plasma fraction. The intensity of the bands with MW of 110 kDa increased by increasing ultrasonic process time (bands a′ - c′). By comparing the protein profile of granules after ionic strength (Figure 5.1) and ultrasonic treatment (Figure 5.2), protein bands with MW 200, 210, 17 and 5 kDa were not appeared in plasma after ultrasound. The number of bands in plasma as the effect of ultrasound remained constant in all ultrasonic treatment (0-10mins). These proteins may be more water soluble compare to other proteins of granule fraction.

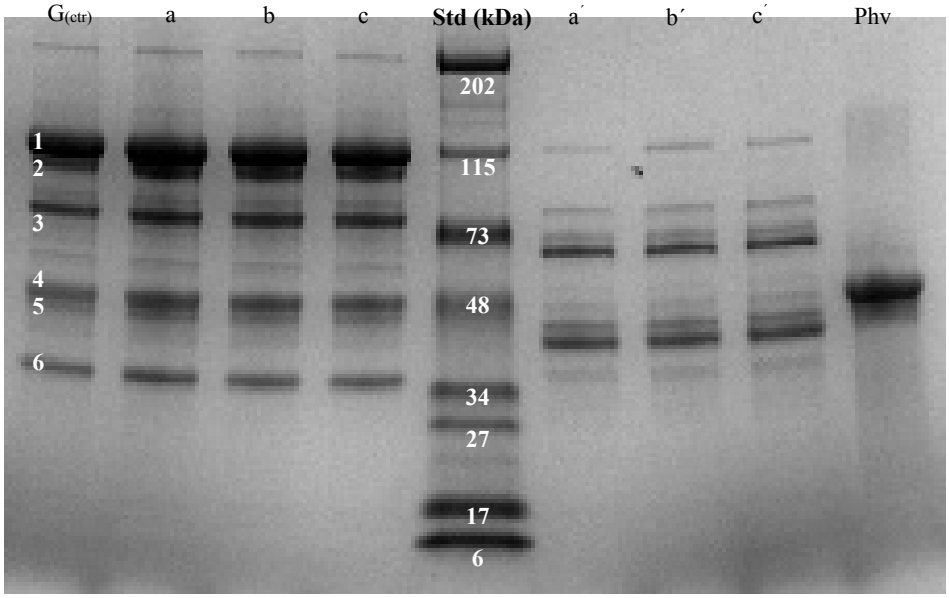

Figure 5.2. Electrophoretic profile of the total proteins of granules after ultrasonic treatment (a-c) and soluble proteins in plasma fraction separated from granules (a´ - c´). Std= MW standard . Phv=commercial phosvitin standard.

5.7. Conclusion

It was demonstrated that under the conditions used (increased ionic strength and ultrasound), the granules are stable structures and their modifications are not possible. Further fractionation of granule is possible by increasing the ionic strength. Moreover, concerning 5-MTHF concentraction in granular fraction, it was observed that by resuspending granules in water, we obtained a granule extract which contains about 20µg folate /g granules. At ionic strength 0.15M NaCl, this amount reached 21 µg folate/g granules extract. However further enhancements were not possible by increasing the salt concentration due to release of folate in plasma fraction. The application of ultrasound treatments provided granules enriched with 21µg/g ganules similar to the concentration calculated for resuspended granule in saline solution with 0.15M NaCl concentration. It was reported that NaCl has the effect of liberating the bound calcium responsible for complexing granular particle through phosphocalcic bridges (Causeret et al., 1992). Bivalent calciums are in fact replaced by monovalent sodium at higher concentration. Phosvitin is the most water soluble protein and liberation of phosvitin by increasing the ionic strengh was accompanied with the detection of 5-MTHF in the solubilized plasma fraction. The novative aproach used in this work confirmed the presence of chemical bonds between the granular protein structure and 5-MTHF in egg yolk granules. Further investigations on the microstructure of granules will provide detailed information in better understanding the association between 5-MTHF and granular protein composition.

CHAPTER 6: Understanding the effect of ionic strength and mechanical treatments on the composition and microstructure of granule separated from hen egg yolk

6.1. CONTEXTUAL TRANSITION

The previous chapter showed that granule has stable structure. By increasing the ionic strength and ultrasound did not significantly affected the folate content of granule. Consequently, this Chapter aims to explore microstructure of granules as the effect of pre-mentioned pre-treatments. Moreover, the high hydrostatic pressure processing was also attempted in order to disrupt granular microstructures and study its folate content.

Results obtained in this part of the project will be submitted by:

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This article is in preparation for submission in Journal of Agriculture and Food Chemistry. Moreover, the results will be presented as two poster presentation in the 12th International Congress on Engineering and Food (ICEF) in Québec City, Canada (June 14-18, 2015).

6.2. RÉSUMÉ

Des travaux antérieurs ont montré que la fraction de granules séparée du jaune d'œuf a été enrichie en acide folique. Des prétraitements tells que l'ajout de sel et l'application d'ultrasons ont été testés pour améliorer la récupération de l'acide folique, mais ont des effets limités dus à la stabilité des granules. Cependant, il a été démontré qu'une concentration en NaCl supérieur à 0.15 M est suffisante pour dissocier la structure granulaire et en libérer le folate dans le plasma. Par conséquent, le présent travail vise à caractériser les effets de prétraitements (force ionique et ultrasons) sur le profil protéique et la microstructure de granules séparés du jaune d'œuf. Un traitement à haute pression hydrostatique (HHP) a également été étudié afin de perturber la microstructure granulaire. Le profil protéiques du granule prétraité et de sa fraction de plasma correspondante a été étudié en utilisant une électrophorèse sur gel 2D. La microstructure des granules a été étudiée en utilisant confocal à balayage laser. Les résultats, montrent que le granule a une structure stable et leur modification n'a pas été possible sous la condition (ultrasons, addition de sel). Les micrographies obtenues par microscopie confocal à balayage laser ont montré un endommagent de la structure de granule après le traitement à haute pression hydrostatique. En outre, une forte concentration de l'acide folique a été détecté dans la fraction de plasma correspondant de HHP traité granule. Le SDS-PAGE a révélé une séparation de phosvitine dans la fraction de plasma où une concentration plus élevée de folate a été mesurée. La perturbation de la structure de granule comme l'effet du traitement HHP facilite la séparation de la phosvitine et le folate dans la fraction de plasma. Ces résultats suggèrent que l'acide folique peut exister sous forme complexée avec des phosvitines dans la structure de granule du granule.

6.3. ABSTRACT

Previous work has shown that the granule fraction separated from egg yolk was enriched with folate. Pre-treatments using salt addition and ultrasound treatments were tested to improve folate recovery but have limited effects due to the granule microstructure stability. Indeed, our investigations revealed that ionic strength more than 0.15 M is enough to dissociate granular structure and release the folate in soluble granular fraction. Consequently, the present work aims at characterizing the effects of pre-treatments (ionic strength and ultrasound) on the protein profile and microstructure of granules separated from egg yolk. High hydrostatic pressure processing was also attempted in order to disrupt granular microstructures. The protein profile of pre-treated granule and corresponding plasma fraction was studied using 2D-gel electrophoresis. The microstructure of granule was studied using confocal laser scanning microscope. Based on results, granule has stable structure and their modification was not possible under the condition (ultrasound, salt addition). The micrographs obtained from confocal laser scanning microscopy showed a granule structure disruption after HHP treatment. Moreover, high concentration of folate was detected in corresponding plasma fraction from HHP treated granule. SDS-PAGE analysis revealed separation of phosvitin in plasma fraction where higher concentration of folate was measured. The disruption of granule structure as the effect of HHP treatment facilitates separation of water soluble phosvitin and folate molecules in plasma fraction. These results suggest that folate may exist as complexes with phosvitin in the granule structure of hen egg yolk.

6.4. INTRODUCTION

Granule fraction of egg yolk provides interesting composition and properties compare to native yolk. This makes its applications attractive to be used as ingredient in food or nutraceutical industries. Numerous studies have been conducted in order to understand the contribution of native structure of granule to its composition, structure and functionality (Anton et al., 2000b; Castellani et al., 2003; Causeret et al., 1992; Causeret et al., 1991; Guilmineau et al., 2005; Laca et al., 2011, 2010; Strixner and Kulozik, 2013).

Seven percent of yolk lipids and 47% of yolk proteins are located in granule fraction. Granules contain high density lipoproteins (HDL), phosvitin and low density lipoproteins (LDL) (Causeret et al., 1991). The protein content of HDL, phosvitin and LDL of granules was reported as 75%, 95%, and 10%, respectively (Powrie et al., 1986). The HDL and LDL constituent of granules incorporated 25% and 88% lipid (on dry matter basis), respectively. Granules, depending on environmental conditions, consist in protein aggregates ranging in diameter from 0.3 μm to several micrometers (Chang et al., 1977).

Granules consist of spherical complexes which form non-soluble HDL-phosvitin complexes linked by phosphocalcic bridges at low ionic strength (Causeret et al., 1991). The presence of numerous phosphocalcic bridges make the granule microstructure very compact, poorly hydrated, and weakly accessible to enzymes and lead to an efficient protection against thermal denaturation and heat gelation (Sirvente et al., 2007). Previous studies indicated the importance of ionic strength on some physiochemical properties (i.e. solubility) of egg yolk granules (Causeret et al., 1992; Causeret et al., 1991). The high concentration of bivalent (Ca^{+2}, Mg^{+2}) and trivalent (Fe^{+3}) cations in granule structure and the presence of phosphoprotein (phosvitin) suggests the presence of ionic bridges between the phosphate group of the phosphoseryl residues of the phosphoproteins (Causeret et al., 1992). However, it was demonstrated that high saline conditions (0.3 M NaCl) dissociated granular structure (Causeret et al., 1991) and increased its solubility to 80% since phosvitin is soluble protein and HDL accordingly acts as soluble protein (Anton and Gandemer, 1997). Moreover, it was also observed that disruption of egg yolk granules by mechanical shear forces (during high pressure homogenization) leads to the formation of granule fragments (Anton et al., 2000a). However, knowledge about the molecular

structure of egg yolk granules and its compositional changes in different milieu conditions is limited. Previous investigations on granule structure were focused on pH adjustment at 4.0 and 6.5 where at pH 4 sedimentation of granules resulted in more compact conformation of the HDL components and a higher amount of bound lipid in granule composition. Due to the negative charge of HDL-phosvitin at pH 6.5, the molecule is more expanded. Furthermore, LDL largely incorporated in HDL granules at pH 4.0 due to the isoelectric conditions and steric interactions (Strixner et al., 2014). In our previous study we observed the importance of the modification of ionic strength on some chemical properties of the granules, i.e., folate and protein content. Our investigations revealed ionic strength more than 0.15 M is enough in order to dissociate granular structure and release the folate in soluble granular fraction.

However, further studies and innovative technical strategies are necessary to better clarify the microstructure and structure-function relationships of egg yolk granules. Recently, the use of high hydrostatic pressures (HHP) for food processing is finding more application in the food industry. Equipment for large-scale production of HHP processed products is commercially available nowadays. HHP is a novel non thermal and quite simple process in which the food (liquid or solid) is subjected to pressures above 100 MPa up to 900 MPa (in commercial systems the pressure is between 400 and 700 MPa). High pressure can disrupt the interactions between food components (San Martin et al., 2002). High pressures can also induce gelation at low temperatures, resulting in gels with properties different from gels obtain with heat (San Martin et al., 2002). HHP was used to study the cold denaturation of proteins. Since proteins at their native state are in highest ordered state thereby, a temperature decrease could not be expected to lead to drastic conformational changes. It was shown that globular proteins undergo a change of protein structure and a disruption at very low temperatures (Privalov, 1990).

The application of HHP and its effect on the retention of the hydrosoluble vitamins B1, B6, and C in a multivitamin model system in egg yolk and in strawberry “coulis” was studied earlier (Sancho et al., 1999). The findings of mentioned study revealed that HHP does not have a significant effect on the retention of either B1 or B6 vitamins (with retention greater than 99%). It was stated that the pressure energy can only disrupt weak bonds, such as hydrophobic and electrostatic ones, while leaving covalent bonds unaffected (Balny et al., 1997; Lullien-Pellerin and Balny, 2002). It has been hypothesized that because HHP does not affect covalent bonds,

small molecules such as vitamins, color, and flavor compounds will remain unaffected after the treatment (San Martin et al., 2002).

The aim of our study was to characterize the effects of pre-treatments on the protein profile and microstructure of granules separated from egg yolk. Moreover, high hydrostatic pressure (HHP) processing was also attempted in order to disrupt granular microstructures.

6.5. MATERIALS AND METHODS

6.5.1. Materials and chemicals

Acetonitrile, methanol and ethanol were HPLC gradient grade and purchased from Sigma-Aldrich (MO, USA); other chemicals were of analytical quality. Chloroform, hydrochloric acid, acetic acid and hydrochloric acid were purchased from Fisher Scientific (NJ, USA). Sodium chloride, (+)-sodium L-ascorbate, and 5-MTHFdisodium salt (90% purity) were obtained from Sigma-Aldrich (MO, USA). Water was purified (≤ -0.1 μS cm-1) using a Milli-Q system of Millipore (LA, USA). Molecular probe Nile Blue A was from Sigma (Steinheim, Germany). Standard reference materials for fatty acid analysis, SRM 1544 and 1946 were purchased from the National Institute of Standards and Technology (NIST).

6.5.2. Preparation of granules

Granules from fresh egg yolks were separated according to Naderi et al. (2014). Briefly, fresh hen eggs were freshly purchased from local market. The eggs were broken manually; albumen was separated from vitelline. The yolk material was diluted with Milli-Q water (1:1 w/w). The granules were separated from diluted yolk by using a tubular bowl centrifuge (CEPA Centrifuge, series LE, Germany) with a maximum centrifugal force of 40,000×g (Naderi et al., 2014).

6.5.3. Ionic strength modification

Granule was subsequently diluted (1:2 w/w) in NaCl solutions of increasing ionic strength (0, 0.1, 0.15, 0.25, 0.55M NaCl). The granule suspensions were mixed using Ultra-Turrax T-25 homogenizer (IKA® Werke Staufen, Germany) for 30s at 6500rpm. The granule suspensions were separated from supernatant (plasma) by centrifugation at 10,000×g for 20 min. At ionic strength of 0.55M NaCl, the entire granule particles were dispersed in saline solution and transformed into a yellow-translucent suspensions. Further separation of granules by centrifugation was not possible.

6.5.4. Mechanical treatments

Ultrasonic treatment and high hydrostatic pressure processing (HHP) were investigated as mechanical treatments applied to resuspended native granule. Portions of native granule were hydrated with ×2 volume of Milli-Q water. The granule solutions were completely dissipated with an ultrasonic instrument (Virsonic 475 Cell Disrupter) in a sealed flask (100 mL) while the temperature of sample controlled by keeping it on ice. An ultrasonic probe with a tip diameter of 7 mm was fitted into the flask and the tip was inserted at the half height of the solution. The ultrasonic process time was set at 0, 5 and 10 min (PULSA^{RTM} pulsing for 15s and relaxing for 10s) and the actual power delivered into the system was 95 W (at 20% amplitude). Thereafter, all granule solutions were centrifuged at 10,000×g for 20 min. The precipitated granules were separated from supernatant and collected in separate vials for further analysis. The supernatant were collected and labeled as plasma for further investigations.

For HHP treatment, 1% w/w granule solution was prepared by re-suspending granule in Milli-Q water and allowed to hydrate overnight at 4°C. The hydrated granule solution was separated in two parts. One part was kept as control and the other part was processed for HHP treatment. For pressurization, granule solution samples were packed in polyethylene terephthalate (PET) bottles of 125 mL leaving no headspace. HHP treatments were performed at 600 MPa during 5 min at room temperature in a discontinuous hydrostatic pressurization unit Hiperbaric 135 (Hiperbaric, Burgos, Spain) with water as pressure transmission medium. Stainless-steel pressure vessel

measures 0.30 m in diameter and 2.20 m in length with a working volume of 135 L. Immediately after pressure treatments, samples were stored at 4°C prior to analysis. The HHP treated granule samples were centrifuged (10,000×g; 20 min; 4°C) to separated granule from supernatant. All the experiments were performed in triplicate on four different batch of fractionated granule.

6.5.5. Compositional analyses

Granules and plasma samples were analyzed in terms of folate, protein, lipid and moisture content according to methods described in Naderi et al. (2014).

Fatty acids were extracted from the egg yolk according to the methods of Folch et al. (1957). The fatty acid composition of samples was determined using standard gas chromatographic techniques of the fatty acid methyl esters (AOAC, 1990), using C17:1 methyl ester as an internal standard.

The cholesterol was extracted and analyzed based on the HPLC method described by Daneshfar et al. (2009) with some modifications. The extracted oil samples were transferred into HPLC vials and injected into the HPLC-UV system (Agilent, Palo Alto, CA) equipped with a fluorescence detector (Waters, Milford, MA). The analytical isocratic RP-HPLC separation was performed by using Kinetex™ C18column (75×2.10 mm; 23^{o}C; flow-rate 0.5 mL/min; UV detection wavelength of 210 nm). The mobile phase was made up of acetonitrile:ethanol (50:50, v/v) and a flow rate of 1 ml/min was used at room temperature. The software Agilent Chemstation was used to control the HPLC system, and to collect and process the chromatographic data.

6.5.6. Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

SDS–PAGE analysis was performed according to the procedure described by (Naderi et al., 2014). Protein solutions (1%) were prepared and further diluted to reach about 25 µg of protein in each gel lane. Samples were diluted (1:1 v/v) in a dissociation buffer consisting of a 0.5 M Tris–HCl pH 6.8, 0.5% bromophenol blue, 35% glycerol, 5% β-mercaptoethanol, 10% (w/v) SDS solution, and heated in a boiling water for 5 min. Electrophoresis were run on precast 4-20% gradient polyacrylamide gels (Bio-Rad, CA) with a migration buffer consisting of a 0.02 M Tris (hydroxymethyl) aminomethane, glycine 5 M and SDS (w/v) 0.1% solution. The proteins were stained using Coomassie blue method which was modified for detection of phosphoproteins (Hegenauer et al., 1977). After destaining in a solution containing acetic acid/methanol/water (10:40:50 v/v/v), the gels were scanned with a ChemiDOC™ XRS (Bio-Rad, CA) system.

Molecular weights were estimated by a molecular weight standard (Precision Plus Protein™ Standards, Bio-Rad Laboratories Ltd, CA).

6.5.7. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Protein profiles of samples from selected conditions were further characterized using 2D-PAGE based on the method described earlier (Naderi et al., 2014). Isoelectric focusing (IEF) step was carried out at 20ºC with an IPGphor system (GE Healthcare Life Sciences) using 13cm Immobiline™ DryStrip gel covering a pH range of 3-10 (GE Healthcare Life Sciences). The protein samples (2mg/mL) were hydrated overnight and desalted subsequently before proteomic analysis. Desalted protein samples diluted (1:1) in sample buffer (urea 7M, Thiourea 2M, chaps 4% v/v, triton-×-100 1% v/v, tris 20mM, H_2O) and loaded onto the strips for first step separation of proteins. The 2nd dimension separation of proteins was achieved on polyacrylamide gels (12%). Analytical gels were consecutively stained using 0.1 M aluminum nitrate added to Coomassie blue (Hegenauer et al., 1977). The gels were de-stained in a solution containing acetic acid/methanol/water (10/40/50 v/v/v).

6.5.8. Microstructure Determination by Confocal Laser Scanning Microscopy (CLSM)

To study the structure of pre-treated granules, samples were analyzed under a confocal laser scanning microscope (CLSM) using a Zeiss LSM 700 (Toronto, ON, Canada) equipped with variable secondary dichroic (VSD) mirror. The molecular probe Nile Blue A was used for staining granules. About 100mg of granule samples was suspended in 0.1 g kg^{-1} Nile blue solution (in demineralized water) at a ratio of 1:10 (w/v) and mixed until the uniformity was obtained. Then the blend was deposited in a cavity slide (containing 25 μL). The samples were examined after one hour diffusion at room temperature with a C-Apochromat 40×/1.2 N.A. water immersion objective. All the fluorescence images have a resolution of 1204 × 1204; 12-bit pixels. Digital image recording was made by using ZEN software program. Final observations were made using laser lines including channel 1: green (ex 488, em 491-629 nm) and channel 2: red (ex 639, em 629-700 nm). The ImageJ2 software was used for image processing.

6.5.9. Statistical analysis

Statistical analysis was performed using a one-way analysis of variance according to the general linear model procedure with least-square mean effects to determine significant differences between treatments. Statistical calculation was investigated using the statistical package Minitab Statistical Software release 16.2.3 for Windows (Minitab®, State College, PA). Confidence intervals were set at 95% ($P<0.05$). All analysis was repeated on four different batches of granule samples. Data were expresse as mean ± standard deviations of triplicate determinations unless specifically mentioned.

6.6. RESULTS

6.6.1. Overall compositional changes induced in granules composition

In our previous study, the application of ultrasound for 10 min and saline solution with concentration of 0.15 M NaCl for pre-treating the granule resulted in extract having 21µg folate/g granule. The comparative composition of granules treated with ultrasound for 10 min and ionic strength at 0.15 M NaCl is presented in Table 6.1. The lipid, protein and SFA content of granules were not significantly different ($p>0.05$) as a function of pre-treatments applied. Higher values were measured for cholesterol (mg/100g) after re-suspending the granules in water and/saline solution at low ionic strength following the mechanical treaments. This was probably caused by rupturing of granules by salt and ultrasound which increased cholesterol concetration and, consequently, its detection. The total saturated fatty acids (mg/g) in granules were not significantly different ($p>0.05$) whatever the pre-treatment procedures. Higher values were measured for folate (µg/100g) content of granule at ionic strength 0.15 M NaCl and ultrasound treatment for 10 min. Regarding the results of previous study (Naderi et al., 2014), the enrichement of egg yolk with natural folate is possible by fractionation method. It was shown that, egg yolk which contains 4µg folate/g dry yolk after fractionation at lab-scale could be enriched with folate in the form of granule with 15µg folate/g granules. Further improvement in folate concentration of granule was possible by using tubular bowl centrifuge at pilot-scale which resulted in granule extract containing 20µg folate/g granules. At ionic strength 0.15 M

NaCl and using ultrasound treatment, granule contained 21µg folate/g granules. Further investigations on the protein compisition of pre-treated granule would elaborate more on the effect of pre-treatment techniques.

Table 6.1. Composition of granule at the optimal pre-treatment conditions

	Sample[1]		
Composition (on dry basis)	**G $_{(C)}$**	**G $_{(0.15\ M)}$**	**G $_{(10\ min)}$**
Lipid (g/100g)	21.8 ± 1.9[a]	22.5 ± 2.5[a]	23.7 ± 0.8[a]
Protein (g/100g)	69.5 ± 2.2[a]	74.6 ± 7.8[a]	72.1 ± 3.0[a]
Cholesterol (mg/100g)	28.5 ± 12.7[b]	70.4 ± 21.7[a]	64.7 ± 8.3[a]
Folate (µg /100g)	1964.9 ± 253.9[a]	2128.2 ± 290.2[a]	2114.9 ± 115.8[a]
SFA (mg/g)	59.8 ± 8.6[a]	65.2 ± 4.1[a]	61.6 ± 1.5[a]

[1] G $_{(C)}$: control untreated granule; G $_{(0.15\ M)}$: granule treated with saline solution at ionic strength 0.15 M NaCl; G $_{(10\ min)}$: granule treated with ultrasound power for 10 min.
[a-b] Values with common superscripts in each rows are not significantly different (P>0.05).

6.6.2. Effect of treatments on protein distribution (2D-PAGE)

The results of the 2D-PAGE are shown in Figure 6.1. Ten groups of spots were identified in the protein pattern of the native egg yolk. The presence of multiple spots in each group was related to different degrees of phosphorylation which was in consistent with the observations of other researchers (Nilsson et al., 2007). The distribution of protein composition of native yolk was in a wide range in terms of molecular weight (17-250 kDa) and p*I* (4-10). The spot 1 and 2 in 2D pattern of native yolk (Figure 6.1-a) was identified as apolipoprotein-B1 and vitellogenin-1, respectively and were also deteted in granule fraction. The spots 3 and 4 (Figure 6.1-b) were identified as phosvitins and lipovitellin-2, respectively and were characteristic of granule protein composition. Spots 7 (Figure 6.1-a) indicates the location of phosvitin and was detected only after staining the gel with aluminium nitrate. The two spots (Figure 6.1-spots 7a and spots 3b, c, e, g, i) were identified at 30 and 35 kDa with p*I* value near to 4. In previous proteomic studies conducted by Nilsson et al. (2007), the molecular weight of 40 kDa was reported for phosvitins. Two groups of spots were presented in the 2D pattern of granule fractions with the same molecular weight of apolipoprotein-B1 and vitellogenin-1 but with lower p*I* value. These multiple spots might be related to the same proteins but with different degrees of phosphorylation. The presence of these spots at molecular weight of 224 kDa,related as

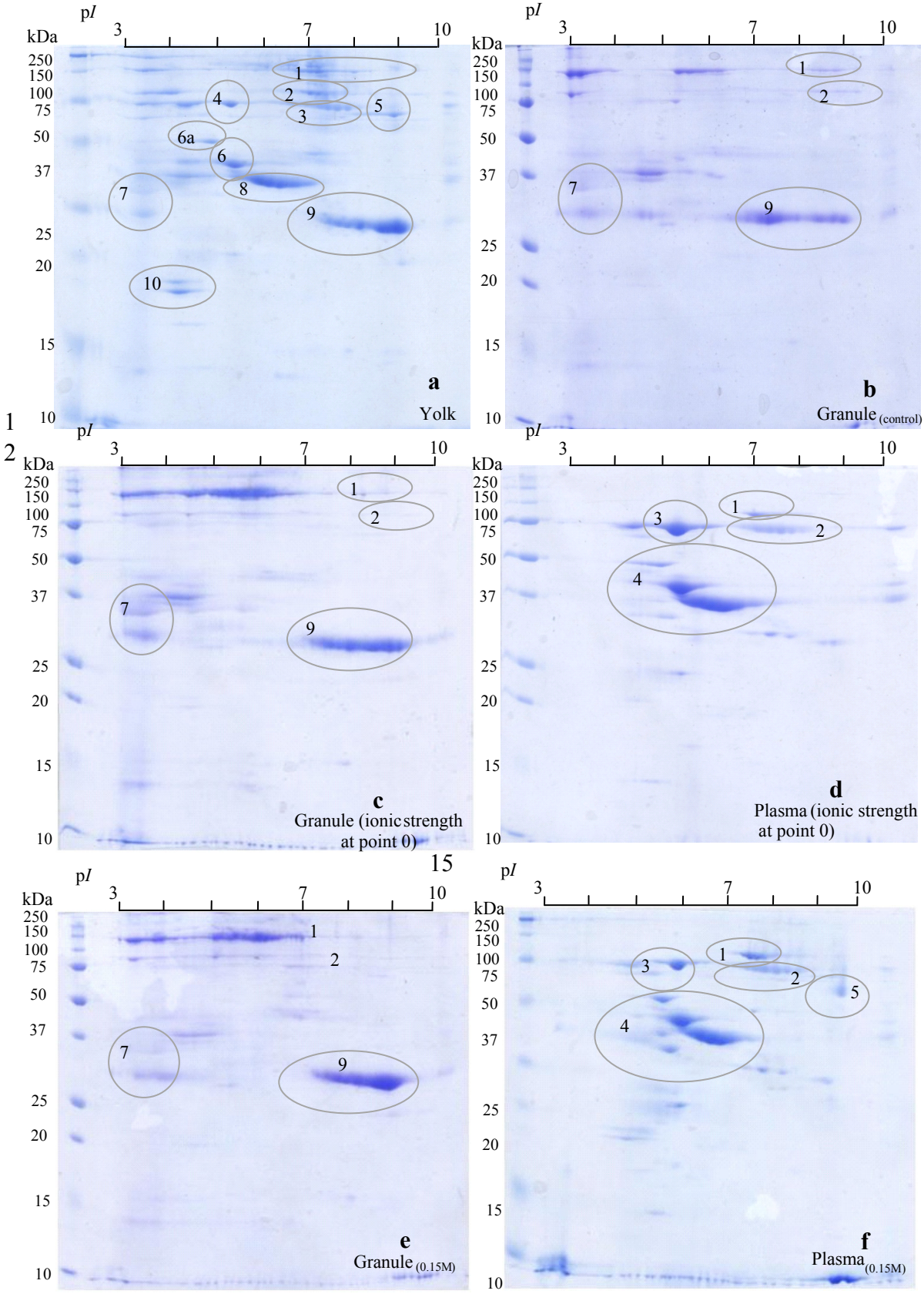
apolipoprotein B1, became more visible by increasing ionic strength at 0.15M NaCl (Figure 6.1-c, e) and by using ultrasonic treatment for 5 and 10 mins (Figure 6.1-g, i). At ionic strength of 0.15M NaCl, the intensity of the spots 1 and 2 in 2D pattern of granule was higher at p*I*<7 (Figure 6.1-g). In granule fractions the location of spots 3 (phosvitin) and 4 (lipovitellin-2) remained unchanged either by increasing ionic strength or ultrasonic treatment. However, the intensity of phosvitin (spot 3) decreased in granule at ionic strength 0.15 M NaCl. The lipovitellin-2 (Figure 6.1-c, spot 4) appeared as multiple spots which were elongated homogeneously between pH 7-9. However, by increasing ionic strength at 0.15M, the intensity of this spot became more evident at pH 9 (Figure 6.1-e). The effect of ionic strength or ultrasonic treatment was more reflected in the 2D pattern of plasma fractions. Four groups of spots were clearly identified in the 2D pattern of plasma fraction (Figure 6.1-d). The location of these spots was identical to the ones in the 2D pattern of egg yolk (Figure 6.1-a). The spots 1 and 2 in Figure-d were located in the same molecular weight/p*I* as vitellogenin-1 and lipovitelin-1 in native yolk (spots 2, 3 in Figure 6.1-a). The presence of spot 3 in plasma was similar to the molecular weight and p*I* of α-livetin in native yolk (Figure 6.1-a, spot 4). A group of 4 spots observed in Figure 6.1-dwas clearly visible in the 2D protein profile of plasma fractions which was detected in the position of spots 6a, 6 and 8 in native yolk. These protein spots were distributed between MW of 50-37 kDa in 2D pattern of yolk and plasma. These proteins were identical to the glycoproteins that were observed in another study (Nilsson et al., 2006). The intensity of these protein spots became more visible at ionic strength of 0.15M NaCl. The spot 6a in the 2D pattern of native yolk (Figure 6.1-a) was not identified however the intensity of this protein increased at ionic strength 0.15M (Figure 6.1-f) and also as the effect of 5 min ultrasonic treatment (Figure 6.1-h). This protein was related to the serum albumin (Nilsson et al., 2006).

By increasing the ionic strength to 0.15M NaCl, the presence of spot 5 was identified in 2D pattern of plasma (Figure 6.1-f). This protein showed MW of 55 kDa and p*I* value of 9.5 which was identical to the spot 5 in native yolk (apolipoprotein B).

The protein spots which were visible in plasma fractions might be related to the LDL which was separated from granule fraction due to the mechanical treatments. It was stated that in the native granules, the HDLs (α- and β-HDL) are linked by phosphocalcic bridges with phosvitin (Anton et al., 2000b). The LDL are entrapped in the granule structure without connection with phosvitin

and HDLs (Anton et al., 2000b; Chang et al., 1977) but the presence of possible protein-protein interactions between granule HDL-constituents and LDL-apoproteins is still unexplored (Strixner et al., 2014). The observation studies of 2D-gel electrophoresis indicated the effect of increasing ionic strength and liberation of LDL from granule structure.

Changes in 2D pattern of plasma were clearly detectable as a function of ultrasound within 10 min (Figure 6.1-j). The spots 2, 3 and 4, identified as vitellogenin-1, lipovitellin-1 and α-livetin, respectively and marked with flesh (Fig 1-j) were observed between isoelectric point of 4 and 10. This might be due to the conformation of proteins resulted as the effect of ultrasound process.


pI
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10
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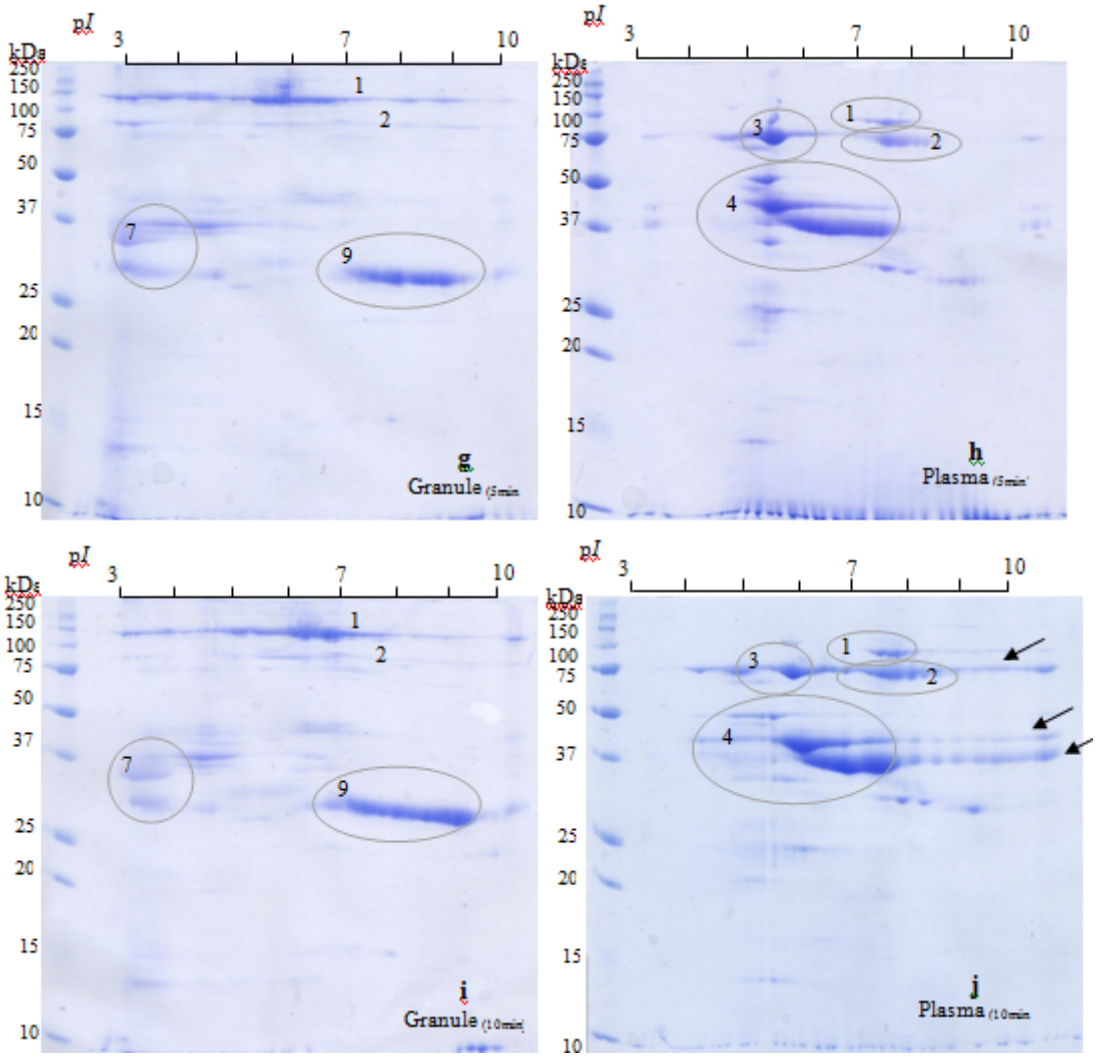
9
10
a
Yolk
b
Granule (control)
c
Granule (ionic strength at point 0)
d
Plasma (ionic strength at point 0)
e
Granule (0.15M)
f
Plasma (0.15M)



Spot	Protein	MW (kDa)[a]	p*I*[a]	Y/G/P
1	Apolipoprotein B, aa1-2017	224	7-8	Y/G
2*	Vitellogenin-1	209	6.5-7.5	Y/G/P
3*	Lipovitellin-1	123	7-8	Y/P
4*, **	α-livetin (serum albumin)	75	5.3	Y/P
5*	Apolipoprotein B, aa2136-2554	55	9	Y/P(0.15M NaCl)
6a	Not identified	-	-	Y/P
6	Truncated serum albumin (aa1-410)	47	5-5.5	Y/P
7	Phosvitin	30-35	4	Y/G
8*, **	β-Livetin (YGP42)	31	5.5-7	Y/P
9*	Lipovitellin-2	28	7-9	Y/G
10	not identified	-	-	Y

*, ** Identifications of protein spots based on Nilsson et al. (2006, 2007).
[a] Compute pI/Mw tool was used to compute the theoretical p*I* and Mw (molecular weight) of proteins. (http://web.expasy.org/compute_pi/)

Figure 6.1. 2D gels of native yolk (a); granules (b); granule at ionic strength 0M NaCl (c); plasma from granule at ionic strength 0M NaCl (d); granule at ionic strength 0.15M NaCl (e); plasma from granule at ionic strength 0.15M NaCl (f); granule after treatment by ultrasonic during 5min (g); plasma from granules after treatment by ultrasonic during 5min (h); granule after treatment by ultrasonic during 10min (i); plasma from granules after treatment by ultrasonic during 10min (j).

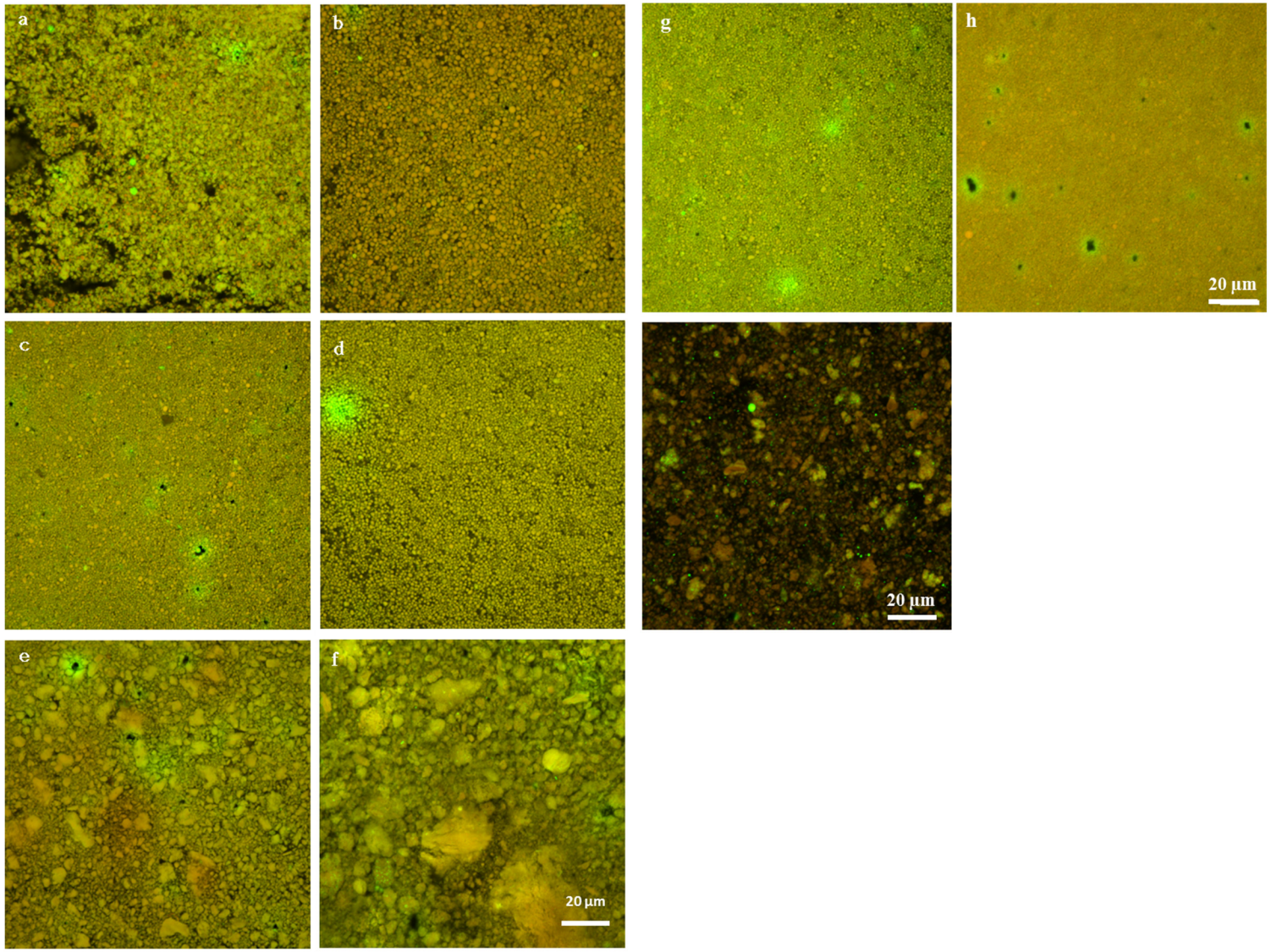
Microstructural changes following treatments

The confocal laser scanning microscope (CLSM) micrographs of fresh egg yolk, untreated (native) and treated (ionic strength variations, ultrasound and HPP treatments) granules are illustrated in Figure 6.2 (a-i). In all figures, where lipids are depicted green and other apolar material red or orange, a combined image of lipids and proteins is presented, In Figure 6.2-a, the protein and lipids are distributed uniformly in fresh egg yolk. The shape of lipid and proteins are irregular with a discontinuous distribution in dye solution. Agglomerated material (protein bodies) as shown in CLSM images in Figure 6.2 (depicted in red/orange), which is obtained by CLSM and visualised with nile blue A. The agglomerated material is surrounded by green dots which are most likely liberated lipids or lipids associated with LDLs. Most of proteins in yolk are organized into micellar and granular structures, together with polar and non-polar lipid moecules (Kiosseoglou, 2003).

The confocal micrograph (Figure 6.2-b) shows the microstructure of untreated granules. The native granules are in the form of microparticles which are the constituents of HDL and phosvitin and bound together by phosphocalcic bridges between their seryl residues and they form insoluble complexes (Anton, 2013). The lipid and protein constituents of granules are homogeniously bound together and lipid embeded in protein structure.

At low NaCl concentration (Figure 6.2c-d) , the destruction of granules led to the formation of an oriented and dense structure where more lipid were released in the granular network. By increasing the salt concentration to 0.25 and 0.55 M (Figure 6.2, f), granules were dissociated and the size of the pseudo-granules were larger than the diameter of ordinary granules. The increase of the diameter could be due to the derease of the number of phosphocalcic bridges due to the loss of phosvitin (Causeret et al., 1991).

Figure 6.2. Confocal micrograph of egg yolk (a), control granule(b), granules treated as the effect of increasing ionic strength (c: 0.1M NaCl, d: 0.15M NaCl, e: 0.25M NaCl, f: 0.55M NaCl), ultrasonic treatment (g: 5min, h: 10min) and HHP (i) prepared at ambient temperature, stained with fluorochrome nile blue. Lipid is represented in the color green and other apolar material, including protein, in red or orange.



After ultrasonic treatment, the granule microstructure became finer and more condensed (Figure 6.2; g-h) with increase in the degree of interconnectivity between granules. No liquid was detectable between granules and many of them were closely packed together. Based on our observations, the stable granule strcture was not modify under the experimental conditions used (modification of ionic strength and ultrasound). Thereby, more drastic condition were applied on granules by using HHP at 600 MPa during 5 min. It was observed that HHP treatment resulted in reorganization of granules. Indeed, figure 6.2-i shows that HHP treatment provoked re-arrangement of granule constituents with the formation of new large aggregates. The granular network is disrupted and they are not connected between them.

The centrifugation of HHP-treated granules resulted in a much less voluminous pellet or granules (G_{HHP}) together with some aggregated material deposit (aggregated granule) on the walls of the centrifugation flask.

Table 6.2 shows the folate concentration of untreated and HHP treated granule and separated plasma fraction. It can be seen that untreated control granule retained higher folate concentration (520.1 ± 2.8 µg/100g) compare to HHP treated granule (15.9 ±0.5 µg/100g). HHP treatment provoked separation of folate in plasma fraction which reached a concentration of 282.3 ± 0.5 µg/100g sample. Consequently, the folate content of the aggregated granule decreased and was measured as 34.5 ± 0.5 µg/100g sample.

Table 6.2. Folate (µg/100g wet sample)

Sample	Folate concentration
G_C	520.1 ± 2.8
Plasma $_C$	42.6 ± 5.9
G_{HHP}	15.9 ± 0.5
Plasma $_{HHP}$	282.3 ± 0.5
Aggregated granule[1]	34.5 ± 0.5

G_C: Control granule; Plasma $_C$: Supernatant control granule; G_{HHP}: HHP treated granule; Plasma $_{HHP}$: plasma separated from HHP treated granule.
[1] Aggregated granule: The granule particles that formed and attached to the wall of tubes after HHP treatment in the form of aggregates.

In order to obtain detailed information concerning granule structure modifications generated after HHP treatment, the protein composition of untreated and HHP treated granules and corresponding plasma fractions was analysed by SDS–PAGE. The electrophoretic profiles are depicted in Figure 6.3. All protein bands can be clearly identified for granule samples. The total granule protein profile consists of 5 bands. HDL apovitellins and phosvitin with apparent molecular weight between 110 and 30 kDa. Phosvitin with molecular weight of 45 kDa was identified in the protein profile of granule. Within plasma profiles, the intensity of the 45 kDa phosvitin protein band is higher as the effect of HHP treatment. The phosvitin was not appeared in the protein profile of untreated plasma separated from control granule. Higher folate concentration was measure in plasma fraction separated from HHP treated granule. Based on these observations, there might be the possibilities of interaction between phosvitin and folate.

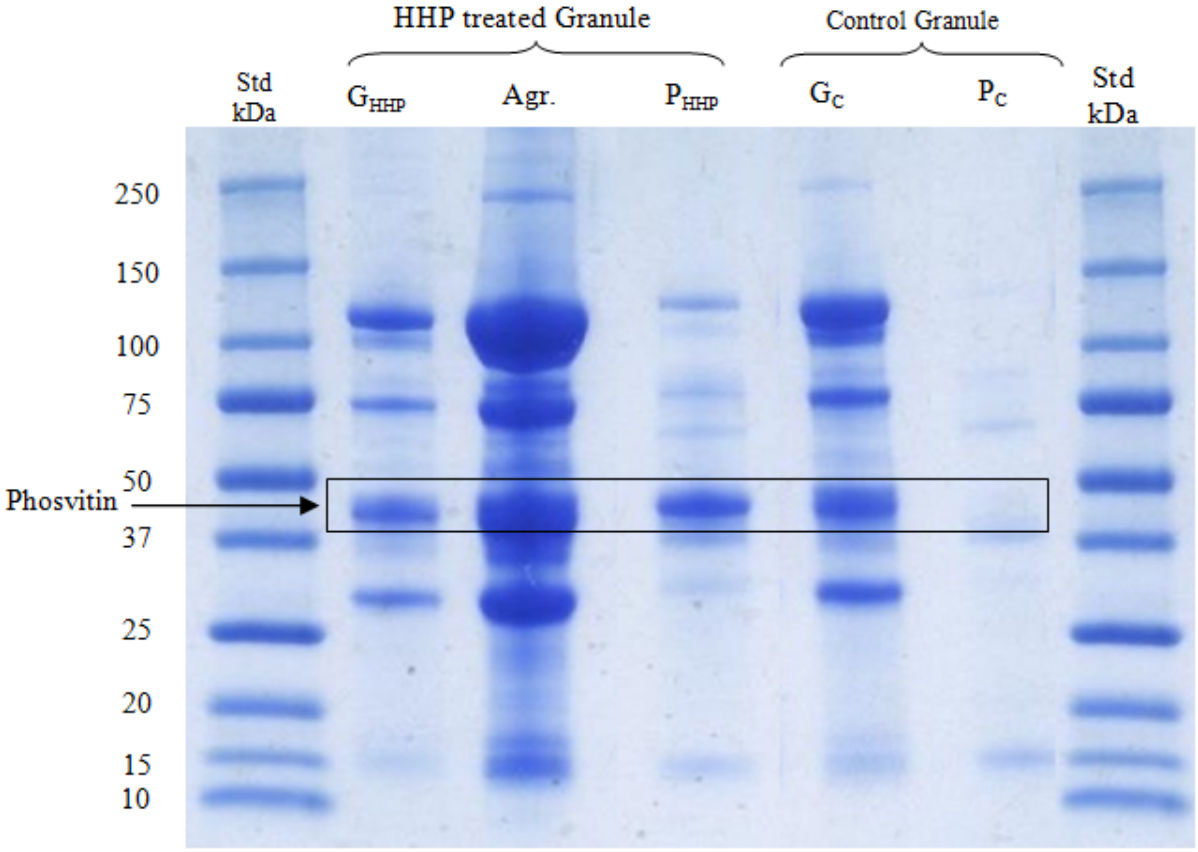


Figure 6.3. Electrophoretic profile of the total proteins of granules after HHP treatment: G_{HHP}; soluble proteins in plasma fraction separated from granules: P_{HHP}; Agr: Aggregated granule; untreated control granule: G_C and soluble proteins in plasma fraction separated from untreated control granule: P_{HHP}- Std= MW standard. Phv=commercial phosvitin standard.

6.7. Discussion

Granule components, in their native condition, are not soluble in water. However, by increasing ionic strength, the disruption of granular network modifies their chemical and physical composition. Granules are composed of HDLs (α- and β-HDL) and phosvitins and contain about 90% of the protein phoshorus and nearly 70% of the calcium in yolk (Burley and Cook, 1961). The existance of phosphocalcic bridges between phosphate groups of three proteins stabilizes the granular network (Causeret et al., 1991). During our previous study (Naderi et al., 2014), we observed that vitamin folate (ligand) is completely associated with granules fraction of yolk and it was further demonstrated that folate can only be separated from granules by increasing ionic strength while mechanical treatment is ineffective. However, marginal changes in folate concentration were obtained by increasing the ionic strength. Higher folate concentration was measured for granule at 0.15 M NaCl ionic strength and ultrasound within 10 min. The composition of granule under the two prementioned conditions was compared with untreated granule. Results showed no differences in the protein, lipid and SFA composition. The cholesterol content of granule was higher as the effect of mechanical treatments compare to untreated granule due to the disruption of granule.

By applying HHP treatment on granule, higher concentration of folate (Plasma $_{HHP} = 282.3 \pm 0.5$) was observed in plasma fraction separated from granule. In addition, results from SDS-PAGE analysis confirmed the release of phosvitin in plasma fraction as the effect of HHP treatment. It can therefore be hypothesized that phosvitin may bind folate. Many studies highlighted the unique polyanionic character of phosvitin (Gaucheron, 2000; Hegenauer et al., 1979) which may be responsible to carry folate in granular structure. However, our 2D-PAGE analyses cannot confirm that phosvitin is a specific candidate responsible to bind folate in granular protein structure.

Phosvitin contain a high proportion of phosphoserin amino acids able to bind calcium. One phosvitin molecule can bind 127 Ca^{2+} ions at pH 6.5 (Anton et al., 2007). The interaction between folate and phosvitin might be from the hydroxyl groups through calcium ions. By adding the sodium chloride, the divalent calcium are replaced by monovalent sodium and the folate was liberated. The affinity constant of sodium towards phosphates is lower than that of

calcium (Causeret et al., 1991). The addition of a large excess of sodium would allow the movement of bivalent cations. Thereby, increasing ionic strength in granule had marginal changes in folate concentration.

Confocal micrographs of pre-treated granule as the effect of increasing ionic strength and ultrasound indicated the stable structure of granules. Indeed, under the experimental condition used, modification of granular structure was difficult.

Based on our results, a schematic representation of granular structure proposed by Strixner et al. (2013; 2014) can be further depicted in Figure 6.4-a. Schematic model of a native (Figure 6.4-a) and disrupted (Figure 6.4-b) granule from egg yolk structure at ionic strength > 0.25 M NaCl is presented. Granules are composed of HDL sub micelles linked to phosvitin through calcium phosphate bridges and trapped LDL spheres in their network. Figure 6.4-b presents the disrupted granule when phosphocalcic bridges are dislocated and phosvitin and β-HDL become solubilised. The LDLs are released from granular network. The folate could be connected to phosvitin via phosphorylation sites in granule network. HDLs (lipovitellines) in granules composed of α- and β-lipovitellin (β-lipovitellin is predominant), phosvitin and LDL (variable amount) which are hold together by ionic forces. The HDLs are organized in a multi level structure.

The refined molecular structure of lipovitellin was described using crystallographic study (Thompson and Banaszak, 2002). The results showed that lipovitellin contains a heterogeneous mixture of about 16% (w/w) noncovalently bound lipid, mostly phospholipid along the walls of the binding cavity (Figure 6.4-a, b). Native granules have compact structure which is not homogeneous and consist of different subfractions (Strixner and Kulozik, 2013) that each granule subfraction incorporated different chemical composition. HDLs are complexed to phosvitins to form the granular structure through phosphocalcic bridges (Figure 6.4-a). The compact structure of granule became more homogenous after mechanical (ultrasound) treatment but no significant change on its chemical composition was observed.

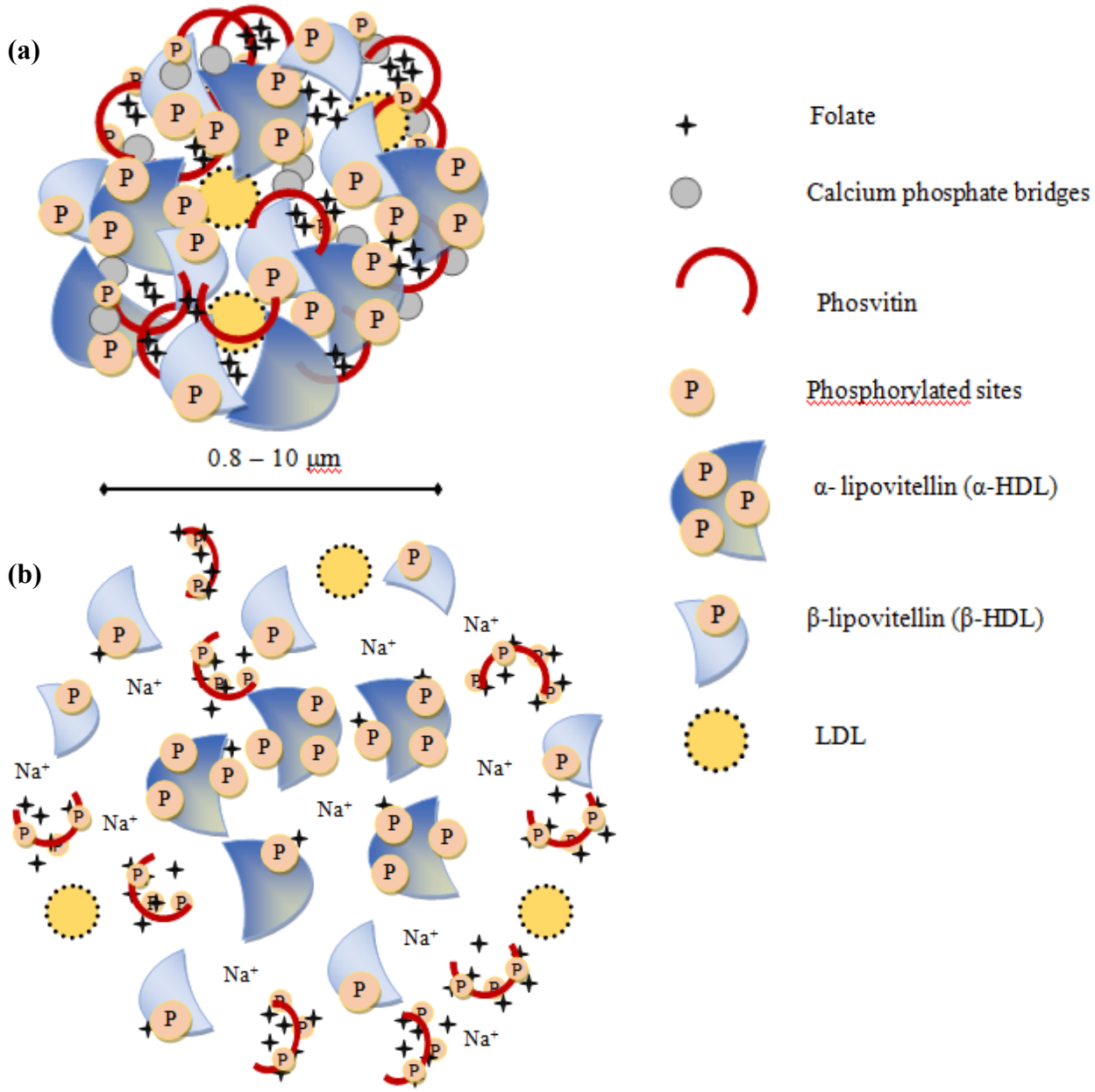


Figure 6.4. Schematic model of a native (a) and disrupted (b) granule from egg yolk structure at ionic strength > 0.25 M NaCl. Composed of HDL sub micelles linked to phosvitin through calcium phosphate bridges and trapped LDL spheres. (b) Disrupted granule when phosphocalcic bridges disrupted and phosvitin and β-HDL solubilised. The LDLs released. Folate connected to phosvitin via phosphorylation sites.

HDLs precipitate in water and become soluble at ionic strength higher than 0.3 M NaCl. β-HDL are more water soluble than α-HDL (Anton, 2007d). The disruption of phosphocalcic bridges (Figuer 6.4-b) occured since monovalent sodium substitute for divalent calcium ions and phosvitin become soluble (Anton et al., 2000a). Since the interaction of folate with the folate binding protein moiety follows a general rule for the folate-protein interaction: the pteridine and p-aminobenzoyl rings are buried into the more hydrophobic interior of the protein with the glutamate tail exposed to the surface of the protein globule (Luka et al., 2007). The dissociation of granular structure and orientation of positively charged sodium ions around glutamic tail of folate faciliate separation of folate in the soluble fraction of granule. Based on the observations we can hypothesize the possibility of occurence of electrostatic interaction between folate, phosvitin, HDLs and Ca^{+2} ions.

6.8. Conclusion

Granule has stable structure and its modification was not possible under experimental condition (increasing ionic strength and ultrasound) used. HHP treatment was effective in destabilizing compact structure of granule and further separation of folate was possible. As the effect of HHP treatment, the water-soluble phosviting and folate were liberated in corresponding plasma fraction. Further studies on the interaction between proteins in the granular structure would allow a better understanding of the granule microstructure and its complex techno-functional properties.

CHAPTER 7: GENERAL CONCLUSION

The overall objective of this research project was to develop a fractionation method of hen egg yolk to produce a natural folate-enriched extract. The general hypothesis behind this doctoral thesis was that *water soluble folate is separable in water soluble fraction of egg yolk by fractionation of egg yolk into its two main fractions of plasma and granule*.

The work carried out allowed us to validate our hypothesis. Fractionation of egg yolk by gravitational separation technique is possible by using only water and folate can be separated in granule fraction of yolk. Fractionation of egg yolk resulted in folate-enriched granule by using a tubular bowl centrifuge at pilot-scale. To some extent, mechanical pre-treatments can result in further fractionation to promote granule folate-enrichments. However, granules had stable structure and their modifications were not possible under the experimental conditions applied. Finally, by using high hydrostatic pressures the intermolecular interaction between HDL, phosvitin and folate (electrostatic or hydrophilic) was disrupted and folate liberated in water soluble fraction of granule.

7.1. Achievements and original contributions

The first objective of this study was to develop and scale-up a sequential separation method using water to fractionate native hen egg yolk and prepare a folate-enriched protein extract. The composition of fractions generated from laboratory-scale and pilot-scale processes were characterized and mass balances were determined.

A simple separation process was used to fractionate egg yolk into plasma and granule fractions through the use of gravitational separation with laboratory- and pilot-scale (tubular bowl centrifuge) centrifuges. The granule fractions at pilot-scale presented with higher protein concentrations (66–69%) and lower lipid contents (20–26%), on a dry basis, as compared to the plasma fraction. The plasma fraction preserved 66–71% of the lipid with the laboratory-scale and 75–77% with the pilot-scale fractionation process. The recovery of protein, based on the raw material (egg yolk), was calculated to be 96% and 89% for the laboratory- and pilot-scale fractionation processes, respectively. Good agreement was achieved between the laboratory- and

pilot-scale centrifugation processes, in terms of chemical composition and recovery of egg yolk components. Granule (28.5 ± 12.7 mg/100g) was also low in cholesterol compare to yolk (89.2 ± 15.6 mg/100g). Granules retained 32% of cholesterol which was 3-fold lower than the cholesterol content of egg yolk. Folate content in plasma and granule fractions was measured using HPLC analysis. Folate analysis revealed that the plasma fraction of egg yolk was devoid of folate, whereas the granule fraction was concentrated by a 3-fold factor in comparison to native egg yolk. It was confirmed by SDS–PAGE and 2D-gel electrophoresis analysis that fractionation process was efficient in separation of proteins in granule and plasma. Considering the recommended daily intake of 400 μg folate for adults, the granule extracted from one egg (58–63 g) by our lab- and pilot-scale separation process can provide 7–8% of daily requirement. The granule fractions separated from egg yolk were rich in folate and low in cholesterol by a 3-fold factor. Based on the results of this part of study, granule is considered as healthier ingredient compare to yolk which can be used in food and nutraceutical products.

To our knowledge, this first attempt in fractionation of yolk and characterizing plasma and granule fraction based on their folate content is totally innovative and gave us important tools for future work.

The second objective of our study was to investigate the potential of increasing ionic strenght or applying ultrasound as pre-treatments to increase its folate content. The effect of these pre-treatments on granular chemical composition was studied.

Granular complex structures form a compact and non-soluble network. Previous studies (Anton, 2013; Castellani et al., 2004; Causeret et al., 1991) showed that the structure of granules closely depends on ionic strength and presence of bi-or polyvalent mineral cations. At an ionic strength over 0.3 M NaCl, the phosphocalcic bridges are disrupted because monovalent sodium replaces divalent calcium (Anton et al., 1997). Thereby, we decided to re-suspend granule in saline solution with increasing ionic strength of 0, 0.1, 0.15, 0.25, 0.55 M NaCl and disrupt the structure of granules and study the possibility of further fractionation.

In recent studies, the application of ultrasonic treatment was successfully used for high molecular proteins and cholesterol degradation in egg yolk (Sun et al., 2011). Thereby, we

decided to apply ultrasound on re-suspended granule in water and study the effect on disruption of granule and the chemical composition of fractions after centrifugation.

Our results evidenced that fractionation of granule is possible by increasing the ionic strength. Indeed, the composition of granule fractions were modified as a function of ionic strength. In order to improve folate concentraction in granular fraction, we observed that at ionic strength 0.15M NaCl granule contained 21 µg folate/g granules. However further enhancements were not possible by increasing the salt concentration due to release of folate in plasma fraction.

Ultrasonic pre-treatment was used to facilitate dispersion of non-soluble granules in water. By increasing the ultrasonic processing time, not significant ($P>0.05$) effect was observed on total protein and lipid content of granules and plasma. Moreover, folate content of granule was not significantly ($P=0.10$) different among treatments. The application of ultrasound provided granules with 21µg of folate/g granules similar to the granule at ionic strength below 0.25 M NaCl concentration. The fatty acid composition of ultrasonic treated granules was increased slightly by increasing the pulsing time period to 10 min. For control granule, the ratio of UFA:SFA and PUFA:SFA was respectively calculated as 1.53 and 0.12. This ratio remained almost unchanged for ultrasonic treated granules at different time points.

The protein profile of granule fraction and separated plasma from re-suspended granules were analyzed by SDS-PAGE. Protein composition of control granule was composed of a total of 6 bands. Five protein bands were assigned to apoproteins of HDL and one protein band with molecular weight of 45 kDa was identified as phosvitin. Addition of sodium chloride to a solution of granules resulted in solubilization of the granular proteins. Protein composition of granules in each treatment was similar to the control granule and the four major apovitellins (HDLs) and phosvitin were clearly visible. The intensity of protein bands decreased across granule profiles by increasing the ionic strength. By comparing protein profile of granules with plasma fraction separated from granule, we noticed that some proteins were solubilized and separated in plasma by changing the salt concentration. The appearance of several protein bands in the plasma fraction after the effect of ionic strength suggests that HDL apoproteins maybe heterodimers made of different size of polypeptides and they dissociated under the conditions applied in this study. By comparing the protein profile of granules as the effect of ionic strength

and ultrasonic treatments, we noticed that the intensity of the protein bands among treatments did not changed for granule fraction but several bands appeared in the plasma separated from granules. The number of bands in plasma as the effect of ultrasound remained constant in all ultrasonic treatment (0-10mins). These proteins may be more water soluble compare to other proteins of granule fraction.

Further investigations on the microstructure of granules were needed to provide detailed information in better understanding the association between folate and granular protein composition.

Based on these observations, we hypothesized that there might be association between protein composition of granule and folate. However, the presence of folate-binding protein in egg yolk has not been confirmed, yet.

The third objective of our study was to provide further information on the composition of granules as the effect of mechanical pretreatments. This study involved the impact of high pressure processing (HHP) on the folate content of the granules. Two-dimensional polyacrylamide-gel electrophoresis (2D-PAGE) was used to visualize the protein profile of disrupted granules. The microstructure of pre-treated granules was studied using Confocal Laser Scanning Microscopy (CLSM).

Granules treated with ultrasound within 10 min and at ionic strength 0.15 M NaCl showed optimal concentration of folate comparing to other conditions. Thereby, we conducted a comparative study on composition of granules treated with pre-treatment conditions. The lipid, protein and SFA content of granules were not significantly different as a function of pre-treatments. Higher values were measured for cholesterol (mg/100g) after re-suspending the granules in water and/saline solution at low ionic strength following the mechanical treatments. This was probably caused by rupturing of granules by salt and ultrasound, in turn making more cholesterol available for easy detection.

The confocal images showed that native granules are in the form of microparticles connected in-between them. At low NaCl concentration, the destruction of granules led to the formation of an oriented and dense structure. By increasing the salt concentration at 0.25, and 0.55 M, granules

were dissociated and the size of the pseudo-granules were larger than the diameter of ordinary granules. The increase of the diameter could be due to the decrease of the number of phosphocalcic bridges. When granules were treated with ultrasound, the microstructure became finer and more condensed with increase in the degree of interconnectivity between granules. Based on our observations, granules have stable structure and their modifications were not possible under the applied experimental conditions. Thereby, we attempted to apply more severe condition as HHP treatment on granules. HHP treatment provoked destruction of granule constituents and they were not connected between them.

The HHP treated granules were characterised based on their folate content and their protein profiles. HHP treatment provoked separation of folate in plasma fraction. Within plasma profiles, the intensity of the phosvitin band was higher as the effect of HHP treatment. Based on our observations there might be the possibilities of interaction between phosvitin and folate.

7.2. Significance of the results

7.2.1. New knowledge on egg yolk granule structure

In the literature, it was mentioned that native yolk granules are composed of large amount of HDL and phosvitin connected together through calcium phosphate bridges. Presence of LDL was reported as being entrapted in HDL-phosvitin network but the existence of protein-protein connections between apoproteins of HDL, LDL is still unexplored.

In this study and for the first time, we showed that the initial folate content of egg yolk was totally recovered in the granule fraction after yolk fractionation by lab- and pilot-scale centrifugations and even after pre-treatments (ionic strength modification or mechanical treatment). Regarding to our observations, we proposed a schematic model for granule structure and we emphasized the presence of two HDL dimers, α-HDL and β-HDL, and existence of inter connection between proteins in granular network through calcium phosphate bridges via phosphorylated sites. Presence of numerous phosphorylated amino acids in protein structure of granules make them highly negatively charged in their native condition. However, presence of high concentration of cations are responsible for formation of bridges between molecules in

granule. In the model, we proposed that the calcium phosphate bridges are the most important elements responsible for inter-connection of the proteins and also folate into the proteins mainly phosvitin.

To improve folate extraction and obtain folate-enriched granule, we increased the granule solution ionic strength and applied mechanical treatments. However, these strategies did no have a significant impact on folate recovery. Moreover, by increasing the ionic strength, the calcium phosphate links were partly disrupted but addition of excessive amount of salt is needed to break the compact structure of granules. The disruption of granular structure is reversible, however the formation of new re-interconnection between apoproteins of HDLs and phosvitin is a subject to be yet explored. The concentration of folate in granule structure appeared to be relevant to the protein content of granules. By applying ultrasound, granule formed more homogeneous structure with no effect on folate separation from protein network structure of granules. Consequently, the granules had very stable structure under the conditions applied and an innovative treatment using high hydrostatic pressure was tested.

It was observed that application of HHP disrupted the granule structure in a way that phosvitin and folate remained in soluble fraction of granules in higher concentration. The application of HHP reported to be effective on disruption of electrostatic and hydrophobic interactions but not on the covalent bonds. Thereby, the soluble fraction of granule had still high concentration of folate after HHP treatment.

Phosvitin is hydrophilic molecule which is highly negatively charged at native condition granule (pH=6.5). Phosvitin contains high amount of ion calcium. Phosvitin and folate might linkes via glutamate tail of folate and phosphate groups of phosphoserin through calcium ions.

7.2.1. New options for processing egg yolk components

The work presented in this thesis is expected to have impact in developing new promising applications to expand the egg-processing industry. Egg yolk has been suffered for bad reputation being high in cholesterol which affected its consumption by consumers. However, egg yolk is a valuable reservoir for natural vitamin folate. Individual constituents of egg yolk are difficult to separate and only plasma and granules can be easily fractionated from yolk at an industrial scale. The results presented in this study demonstrated the possibility to obtain granule fraction enriched with folate after fractionation of egg yolk through a green and toxic-free process. Moreover, the granules were characterized being low in cholesterol and saturated fatty acid and being high in protein.

Granule has compact structure being high in phosphoproteins. The presence of numerous phosphocalcic bridges in the structure of granule between phosvitin and HDL are responsible for compact and non-soluble structure. Previous studies have shown the effect of increasing ionic strength in disruption of phosphocalcic bridges in granule structure. We were attempted to apply pre-treatment procedures in order to affect the solubility of granules and improve folate concentration in final separated granule fraction. Our results showed that by increasing ionic strength, the changes in folate concentration of granule were marginal. The ultrasound can be used to influence disruption of granules but further changes in folate content of granule are not possible. The application of HHP treatment is an interesting solution for disrupting granule structure and liberating folate in soluble fraction of granule. SDS-PAGE analysis revealed presence of phosvitin in plasma fraction after HHP treatment on re-suspended granules. Based on our results, electrostatic or hydrophobic interactions could be responsible in holding folate, phosvitin and HDLs together in granule fraction. These results showed that there are possibilities to develop new uses for granules from egg yolk in food or pharmaceutical applications.

7.1. Research perspectives

Based on the results obtained in chapter 5, increasing ionic strength to 0.15 M NaCl and using ultrasound within 10 min improved the folate concentration in granule. However, optimization of the process by combining pre-treatment factors and fractionation at larger scale would be an interesting challenge.

SDS-PAGE results gave well-defined protein bands for granule fraction after treatments with salt and ultrasound. Differences between proteins bands separated from granule after ultrasound were not observed. However, the total number of soluble proteins separated from granule after increasing ionic strength increased. Mass spectrometry analysis could be used to identify the proteins.

In chapter 6, HHP treatment at 600 MPa within 5 min was tested to increase folate content of granule. The resulted disrupted granule presented a soluble phase after centrifugation which was mainly composed of phosvitin and folate. There is a possibility of electrostatic interaction between folate and phosvitin via calcium ions. However, further study of the mechanism underlying interaction between phosvitin and folate could be important for the development of protein based carrier systems for the delivery of bioactive molecules. The interaction of folate with phosvitin can be studied using fluorescence, absorption spectroscopy and circular dichroism. Furthermore, there is a need to develop and scale-up folate-enriched granule extract by using HHP approach coupled with non-toxic extraction method.

Folate is mainly recognized as being unstable and thereby application of natural folate in food industry was uncertain. However, our development fractionation process at pilot-scale revealed that folate is stable when it was detected in association with protein content of granule. Our results provided highly promising evidences concerning the recovery of high-concentration folate extract from hen egg yolk. Our fractionation technique is also clean but it generates plasma as co-product that is still usable in food formulation. Such applications still need to be developed before the technology can be viable at commercial scale.

In order to develop natural health products from proposed fractionation technique, it will first be necessary to study the bioavailability of folate from enriched-granule (fresh and freeze-dried) compared to fresh egg yolk by conducting animal study. Also clinical data on tolerance, safety and efficacy of the extracts will be required before such product can reach commercial scale.

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