Antioxidants in Chicken Egg Yolk: Effects of Cooking, Storage and Gastrointestinal Digestion

by

Liyana Arachchilage Chamila Koushalya Nimalaratne

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science

University of Alberta

ABSTRACT

Eggs are one of the most nutritious and economically affordable foods. They contain high quality proteins and lipids and serve as an important source of vitamins and minerals. Despite the nutritional importance, the egg industry is vulnerable to the consumers' perception about egg cholesterol and associated risk of cardiovascular diseases. A correlation between egg cholesterol and risk of cardiovascular diseases is not supported by many clinical studies; findings from recent research, however, suggested that egg contains many bioactive components that may provide health benefits beyond basic nutrition.

Oxidative stress is associated with onset and development of various diseases including cancers, and cardiovascular and neurodegenerative diseases etc. Antioxidants in the diet are thought to play a protective role against oxidative damage. In comparison to extensive research on antioxidants from plants (fruits, vegetables, cereals, herbs etc), there is limited research on egg antioxidants.

The Canadian egg industry uses corn and wheat as their main feed ingredients. It was not known if the phenolic compounds in these cereals could be transferred from feed into the egg yolk. Moreover, the potential of chicken eggs as an antioxidant food commodity has not been fully explored. We aimed to test two hypotheses: 1) antioxidant compounds present in hen's feed may contribute to antioxidant activity of egg yolk, and 2) domestic cooking, storage and gastrointestinal digestion would affect the antioxidant activity of egg yolk.

In the first study, our results showed that phenolic acids in feed could not be transferred into eggs; for the first time, we identified that two aromatic amino acids, tryptophan and tyrosine, are the major contributors to the antioxidant property of egg. The antioxidant activity, based on

oxygen radical absorbance capacity (ORAC), DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging and ABTS (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) radical scavenging assays, and the contents of aromatic amino acids were significantly reduced by all cooking methods (boiling, frying and microwaving).

The effect of cooking methods on yolk carotenoids, a well-known group of antioxidants, was evaluated. A LC-(APCI)-MS/MS method was used to identify and quantify all-*E*- and *Z*- isomers of lutein, zeaxanthin, canthaxanthin and β-apo-8'-carotenoic acid ethyl ester in fresh and cooked egg yolks. Both fresh and cooked yolks showed similar carotenoid profiles except the content of *Z*-isomers increased while that of *E*-lutein reduced in cooked samples; the total loss of carotenoid ranged from 6 to 18% in different cooking methods.

A stepwise extraction protocol was developed to determine the total antioxidant activity of egg yolk. Samples from raw and cooked egg yolks were extracted to obtain four fractions containing carotenoids, vitamin E, free amino acids (FAAs), and phosvitin. The antioxidant activity of each fraction was determined by ORAC and ABTS assays. FAAs contributed to approximately two thirds of total antioxidant activity. The effects of simulated retail storage conditions and cooking on the antioxidant activity as well as the aromatic amino acids and carotenoids in general table eggs, omega 3/lutein enriched eggs, and eggs from heritage chicken breeds were evaluated. Different types of eggs did not differ in their FAA, ORAC and malondialdehyde (MDA) contents. Six weeks of storage at refrigerated temperature did not change the ORAC value and the contents of FAAs, carotenoids, and MDA in egg yolk. Boiling and frying significantly reduced the ORAC value and the contents of FAAs and carotenoids while the MDA level increased.

An *in vitro* model (TIM-1) was used to investigate the effect of simulated gastrointestinal digestion on antioxidant activity and carotenoid profile of egg yolk. Bioaccessibility, but not digestive stability was significantly affected by the method of cooking. No *trans-cis* isomerization of carotenoids was observed while scrambled eggs showed a significantly lower bioaccessibility compared to that of boiled eggs. Gastrointestinal digestion significantly increased the ORAC value of eggs. FAA analysis revealed that digestion resulted in a 17-fold increase in FAAs in whole egg and 4.5 to 6-fold in egg yolk. Free aromatic amino acids contribute to the antioxidant activity; however, they were not the major contributing factor.

The findings of this thesis establish the primary evidence of egg as an antioxidant food commodity; the antioxidant activity of egg is not affected by storage, reduced during cooking but significantly increased during gastrointestinal digestion.

PREFACE

This thesis contains original work done by Chamila Nimalaratne and has been written according to the guidelines for a paper format thesis of the Faculty of Graduate Studies and Research at the University of Alberta. The concept of the thesis originated from my supervisor Dr. Jianping Wu and the research was funded by the grants from Egg Farmers of Canada (EFC), Alberta Egg Producers (AEP), the Agriculture and Food Council, which is responsible for delivering Agriculture and Agri-Food Canada's Advancing Canadian Agriculture and Agri-Food (ACAAF) Program in Alberta, Food for Health Initiative (Vitamin Fund) of the Faculty of Agricultural, Life & Environmental Science of the University of Alberta, Burnbrae Farms Limited, Poultry Industry Council and Natural Sciences and Engineering Research Council (NSERC) of Canada to Dr. Wu.

The thesis is consisted of nine chapters: Chapter 1 provides a general introduction and the objectives of the thesis. Chapter 2 is literature review regarding the oxidative stress, dietary antioxidants and antioxidative properties of egg components;

Chapter 3 has been published as “Free aromatic amino acids in egg yolk show antioxidant properties” in *Food Chemistry* and Chapter 4 has been published as “Effect of domestic cooking methods on egg yolk xanthophylls” in *Journal of Agricultural and Food Chemistry*. I was responsible for the literature search relevant to above studies, designing and performing the laboratory experiments, data analysis and writing the first drafts. Drs. Jianping Wu and Andreas Schieber contributed to experimental design, data interpretation and preparation and submission of manuscripts. Mrs. Daise Lopes-Lutz provided substantial assistance in conducting LC-MS/MS analysis, data interpretation and preparing the manuscripts.

Chapter 5 describes “Stepwise Extraction of Antioxidants from Egg Yolk”. I was responsible for designing and performing laboratory experiments, data analysis and writing the first draft of the chapter. Chapter 6 is in preparation for submission as “Effects of storage and cooking on the antioxidant capacity of different types of chicken egg yolk”. I was responsible for compilation of the relevant literature, designing and conducting experiments, data analysis and writing the first draft of the manuscript. Dr. Jianping Wu contributed to experimental design, data interpretation and the composition of the Chapters 5 & 6.

Chapter 7 and 8 contains experiments conducted at Institute of Nutrition and Functional Foods (INAF), Université Laval, Quebec city, QC under the collaboration with Dr. Sylvie F. Gauthier. Mrs. Patricia Savard contributed by designing and performing the simulated gastrointestinal digestions of egg samples using TIM-1 and collecting the samples. My contributions were to assist in designing and performing the TIM-1 digestions and sampling. The rest of the experiments were conducted in our laboratory under the supervision of my supervisor Dr. Jianping Wu. I was responsible for conducting experiments, data analysis and drafting the manuscripts. Drs. Jianping Wu and Sylvie F. Gauthier contributed to the experimental design and results interpretation. In addition, Drs. Jianping Wu, Sylvie F. Gauthier, Andreas Schieber and Mrs. Patricia Savard also helped in manuscript edits. Chapter 7 and 8 are in preparation for submission as “Effect of different cooking methods on bioaccessibility and digestive stability of egg carotenoids as studied in an *in vitro* gastrointestinal system (TIM-1)” and “Antioxidant activity of egg yolk increased after simulated gastrointestinal digestion using the TNO gastrointestinal model (TIM-1)” respectively.

DEDICATION

Dedicated to my beloved parents,

Nimalaratne Liyanaarachchi and Rohini Dasanayake

ACKNOWLEDGEMENTS

There are many people who have encouraged and supported me throughout my graduate program and who I am forever grateful to. First and foremost, I would like to express my heartiest gratitude to my supervisor Dr. Jianping Wu for providing the opportunity to pursue my graduate studies and for his enormous support, encouragement and guidance given throughout the program. I am deeply grateful to my co-supervisor, Dr. Andreas Schieber for his invaluable guidance, motivation and support in both academically and socially during my studies. I am also very thankful to Dr. Michael Gänzle, for his support as the supervisory committee member. I would like to express my sincere thanks to Dr. James House for accepting to be the external examiner for my PhD defense and to Dr. Ellen Goddard for serving as an internal/external examiner.

The financial support received throughout my doctoral program by Natural Sciences and Engineering Research Council of Canada (NSERC), Egg Farmers of Canada (EFC), Alberta Egg Producers (AEP), the Agriculture and Food Council, Burnbrae Farms Limited, Poultry Industry Council and Food for Health Initiative (Vitamin Fund) of the Faculty of Agricultural, Life & Environmental Science of the University of Alberta, the Faculty of Graduate Studies, University of Alberta is highly appreciated.

I am very thankful to Dr. Daise Lopes-Lutz for her guidance, help and support with chromatographic and mass spectrometric analysis, and I treasure the friendship we shared over the course of the program. I am really grateful to Dr. Sylvie Gauthier and Mrs. Patricia Savard for their great support during my experiments in Institute of Nutrition and Functional Foods (INAF), University of Laval.

I am deeply grateful for all the support given by the Graduate Program Administrator of the Department of Agricultural Food and Nutritional Sciences (AFNS), Mrs. Jody Forslund. The technical support of Garry Sedgwick and Will Cornet is also greatly acknowledged.

I would like to express my sincere thanks to my fellow graduate students, both past and present, from Dr. Wu's and Dr Schieber's laboratories: Dr. Shengwen Shen, Dr. Sunjong you, Dr. Bo Lei, Dr. Jiapei Wang, Dr. Morshedur Rahman, Dr. Aman Ullah, April Milne, Maria Offengenden, Alexandra Acero, Kaustav Majumder, Li Sen, Sahar Navidghasemizad, Mejo Remanan, Jiandong Ren, Wenlin Yu, Elizabeth Mudge, Yuchen Gu, Justina Zhang, Qiyi Li, Yussef Esparza, Xiahong Sun, Ali Akbari and Forough Jahandideh. I am thankful for their numerous support and company and I cherish the memories of good times we shared together.

I am blessed to have such wonderful friends who I consider as my family in Edmonton. Nimesh & Dhanuja, Chamila & Nilu, Prasanna & Sumali, Wijaya & Thushani, Dinuka & Kethmi will always be remembered for their love and support.

I would not have come so far without the support of my family. I am so grateful for my loving parents, my sisters: Kalpana and Madhavi and my brother, Madara, for their endless love, caring and for always been there for me throughout my life. I would also like to thank my loving parents-in law and brother and sister in-law Tharaka and Amila for their love and support. Words cannot express my gratitude towards my loving husband Nandika Bandara and my little girl Chathuli for their unconditional love, care, and understanding and for always being by my side through thick and thin.

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CHAPTER 1- General Introduction

Chicken (*Gallus gallus domesticus*) egg consists of outer shell with inner membranes, egg white and egg yolk representing approximately 9.5%, 63% and 27.5%, respectively (Li-Chan, Powrie, & Nakai, 1995). On average, a large egg (~ 53 g of edible portion) contains 7 g of proteins, which are distributed in white and yolk and 6 g of fats, almost exclusively found in egg yolk. It also contains ~1% carbohydrates, vitamins and minerals (Sugino, Nitoda, & Juneja, 1997). The main constituents of egg white are water (~90%) and proteins (~10%) such as ovalbumin, ovotransferrin, ovomucoid, ovomucin, lysozyme etc. Approximately 50% of yolk is water while lipids and proteins account for 32.6% and 16% respectively. The proteins in the yolk exist as lipoproteins (low and high density), globular proteins (livetins) and phophoproteins (phosvitin) and some other minor proteins. The lipid fraction is made of 62% triglycerides, 33% phospholipids and less than 5% cholesterol (Anton, 2007).

Eggs are one of the most nutritious and economically affordable foods. Egg proteins are considered the highest quality and used as the "gold standard" for protein quality assessment. Phosphatidylcholine makes around 76% of phospholipids and has been recognized as an important nutrient for brain development, liver function, and cancer prevention (Zeisel, 2004). Eggs are a considerable source of vitamins and minerals. One egg provides more than 10% of the recommended daily intake of fat-soluble vitamins (A, D, E, K) and several of the vitamin B group (Seuss-Baum, 2007). Eggs also contain significant amounts of phosphorus, selenium, iron, zinc and most of the other minerals in varying amounts (Seuss-Baum, 2007).

However, the consumption of eggs is affected largely by the general public perceptions about the presence of cholesterol in egg yolk and associated risk of cardiovascular diseases. Egg consumption in relation to serum cholesterol levels and cardiovascular diseases was one of the

major research motivations during the past few decades yet failed to prove any direct association between egg consumption and cardiovascular diseases (Hu, 1999; Lee & Griffin, 2006; Nakamura *et al*., 2007). In contrast, many recent research activities have demonstrated that egg is a rich source of many biologically active compounds, beyond supplying basic nutritional requirements, including antihypertensive, antioxidant, antimicrobial, immunomodulating, and anticancer properties (Hatta, Kapoor, & Juneja, 2008; Huopalahti, López-Fandino, Anton, & Schade, 2007; Kovacs-Nolan, Phillips, & Mine, 2005; Mine & D'Silva, 2008).

The generation of free radicals (reactive oxygen species, ROS, and reactive nitrogen species, RNS) as a result of normal cellular metabolism (Valko *et al*., 2007) is essential to a number of cellular signaling systems and provides protection against pathogens (Halliwell, 1994). However, an imbalance between free radical formation and antioxidant scavenging can cause oxidative stress and damage to lipids, proteins, or DNA, inhibiting their normal function, which is associated with many chronic diseases, for example, atherosclerosis, cancer, diabetes, aging, Alzheimer's disease and other degenerative diseases in humans (Halliwell, 1996; Valko *et al*., 2007). Intake of antioxidants via foods or supplements is believed to be effective in reducing oxidative stress (Halliwell, Murcia, Chirico, & Aruoma, 1995). The presence of antioxidants has been extensively studied in plants but there is limited work on animal derived antioxidants (Adom, Sorrells, & Liu, 2005; Li, Pickard, & Beta, 2007; Liu, 2007). The well-known antioxidants in eggs include proteins and their derived peptides (Chen, Chi, Zhao, & Lv, 2012; Dávalos, Miguel, Bartolomé, & López-Fandiño, 2004; Huang, Majumder, & Wu, 2010; Park, Jung, Nam, Shahidi, & Kim, 2001; Sakanaka, Tachibana, Ishihara, & Raj Juneja, 2004; Sakanaka & Tachibana, 2006; Xu, Katayama, & Mine, 2007; You, Udenigwe, Aluko, & Wu, 2010; Young, Nau, Pasco, & Mine, 2011). Carotenoids like lutein and zeaxanthin (Thurnham,

2007; Trevithick-Sutton, Foote, Collins, & Trevithick, 2006), vitamin E (Surai, 2000), selenium (Surai, 2000), and isoflavones, which transferred into the eggs from plants, were also reported in eggs (Kuhnle *et al*., 2008; Saitoh, Sato, Harada, & Matsuda, 2004).

The Canadian egg industry uses corn and wheat as their main feed ingredients which are rich in antioxidant phenolic compounds such as ferulic syringic, vanillic, and *p*-hydroxybenzoic acids (Dewanto, Wu, & Liu, 2002; Liyana-Pathirana & Shahidi, 2006; Zaupa *et al*., 2014). It was not known if some of these antioxidant phenolic compounds could be transferred from feed into the egg yolk. Moreover, although the antioxidant properties of individual egg yolk components have been investigated, the potential of whole egg yolk as an antioxidant food commodity has not been fully explored. In this thesis we aimed to test two hypotheses that antioxidant compounds present in hen's feed may contribute to the antioxidant activity of egg yolk, and that domestic cooking, storage and gastrointestinal digestion could change the antioxidant activity of egg yolk. In order to investigate the above hypotheses, the following objectives were addressed:

1. To determine the antioxidant activity of corn-fed and wheat-fed egg yolk under different types of domestic cooking methods.
2. To develop a stepwise extraction method to identify the compounds contributing to the total antioxidant activity of egg yolk.
3. To investigate the changes in egg antioxidants during normal storage conditions.
4. To determine the effect of simulated gastrointestinal digestion on activity and bioaccessibility of egg yolk antioxidants.

CHAPTER 2 - Literature Review

2.1. Free Radicals, Oxidative Stress and Human Diseases

2.1.1. Free Radicals and Other Reactive Species in Human Body

By definition, a free radical is any molecule or an atom capable of independent existence that contains one or more unpaired electrons (Halliwell, 1994). The hydrogen atom, with one proton and a single electron, is considered the simplest form of free radical. Radicals can interact with another radical making a non-radical, which is usually less reactive, except in some cases; for example, in reaction I, a non-radical peroxynitrile ($ONOO^-$) formed by reacting superoxide radical ($O_2^{\cdot-}$) with nitric oxide radical ($NO^{\cdot}$) is more damaging to human tissues than either of the parent radicals (Valko *et al*., 2007).

$$O_2^{\cdot-} + NO^{\cdot} \longrightarrow ONOO^- \quad (I)$$

Interaction between a free radical and a non-radical biological molecule leads to the formation of a new radical, initiating a chain of oxidation reactions such as lipid peroxidation (Bochkov *et al*., 2010). In reaction (II), hydroxyl radical ($OH^{\cdot}$) reacts with lipid molecule (L–H) to form water and lipid radical ($L^{\cdot}$), which then can react with oxygen to form lipid peroxyl radical ($LOO^{\cdot}$, reaction III). Lipid peroxidation has been associated with several pathological conditions including atherosclerosis, Alzheimer's disease etc (Yin, Xu, & Porter, 2011).

$$L–H + OH^{\cdot} \longrightarrow H_2O + L^{\cdot} \quad (II)$$

$$L^{\cdot} + O_2 \longrightarrow LOO^{\cdot} \quad (III)$$

Reactive species can be divided into several categories. Reactive oxygen species (ROS) are defined as oxygen-containing reactive species. They include superoxide, hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hydroxyl radical ($OH^{\cdot}$) and organic peroxides etc. (Nathan & Ding, 2010). Reactive nitrogen species (RNS) include nitric oxide ($NO^{\bullet}$), nitrogen dioxide radical ($NO_2^{\bullet-}$), and other oxides of nitrogen or nitrogen-containing reactive species (Fubini & Hubbard, 2003; Nathan & Ding, 2010). The generation of ROS & RNS in cells is tightly regulated and involve many functions ranging from cell homeostasis to cell death (Finkel, 2011). Mitochondria produce cellular ROS as a consequence of oxidative phosphorylation (Balaban, Nemoto, & Finkel, 2005). It is suggested that during mitochondrial electron transfer chain, 1-2% electrons can be leaked and react with oxygen to generate $O_2^{\bullet-}$, the primary ROS, which is then converted to H_2O_2 (Poli, Leonarduzzi, Biasi, & Chiarpotto, 2004). Another important source of ROS is the family of membrane proteins called NADPH (reduced nicotinamide-adenine di-nucleotide phosphate) oxidases (Nox), which produce superoxide and hydrogen peroxide (Brown & Griendling, 2009). Endoplasmic reticulum membranes containing cytochrome *p*-450 and *b*5 enzymes also produce H_2O_2 triggered by the Ero1(Endoplasmic Reticulum oxidoreductin 1), a glycoprotein (Gross *et al*., 2006). The hydroxyl radical $OH^{\bullet}$ is a very reactive radical with a half-life of approximately 10^{-9} seconds (Valko *et al*., 2007). The release of transition metal ions such as copper or iron ions from iron–sulphur clusters, haem groups or metal storage proteins can catalyze the conversion of $O_2^{\bullet-}$ and/or H_2O_2 to $OH^{\bullet}$ (Liochev, 2013).

In most cases, ROS are made purposely by endogenous sources to regulate various physiological functions. One significant function of ROS such as superoxide anions and hydrogen peroxide is to initiate biological processes as the intracellular second messengers (Finkel, 1998). It is shown that production of H_2O_2 is essential for activation of numerous signal transduction pathways,

particularly those mediated by tyrosine kinases (Salmeen *et al*., 2003). Recent findings suggest that ROS are involved in cell migration in the wound healing process (Niethammer, Grabher, Look, & Mitchison, 2009) and stem cell proliferation (Le Belle *et al*., 2011). ROS also play a role in innate immunity. Upon recognizing a pathogen or foreign particle, phagocytic cells such as the neutrophils start a series of reactions called respiratory burst, during which oxygen is converted to $O_2^{\cdot-}$ by NADPH oxidases killing pathogens and microorganisms (Decoursey & Ligeti, 2005; Fubini & Hubbard, 2003; Valko *et al*., 2007). In addition, they are involved in limiting and terminating the specific immune/inflammation responses, and regulating the circadian rhythm in the absence of transcription or translation (Dickinson & Chang, 2011; Nathan & Cunningham-Bussel, 2013). Nitric oxide, $NO^{\bullet}$, a free radical generated by specific nitric oxide synthases (NOSs) is an important signalling molecule in regulating the vascular tone, involved in neurotransmitter function in both the central and peripheral nervous systems, and also a mediator of cellular defence (Bergendi, Beneš, Ďuračková, & Ferenčik, 1999). In addition, $NO^{\bullet}$ interacts with the mitochondrial system to regulate cell respiration and enhance the generation of reactive oxygen species, thus playing an indirect role in cell survival or death (Moncada & Higgs, 2006). Interaction between $NO^{\bullet}$ and other molecules generates RNS, which can induce excessive lipid peroxidation and formation of reactive aldehydes such as - malondialdehyde (MDA; Radi, Beckman, Bush, & Freeman, 1991). Formation and breakdown pathways of ROS/RNS are illustrated in Figure 2.1.

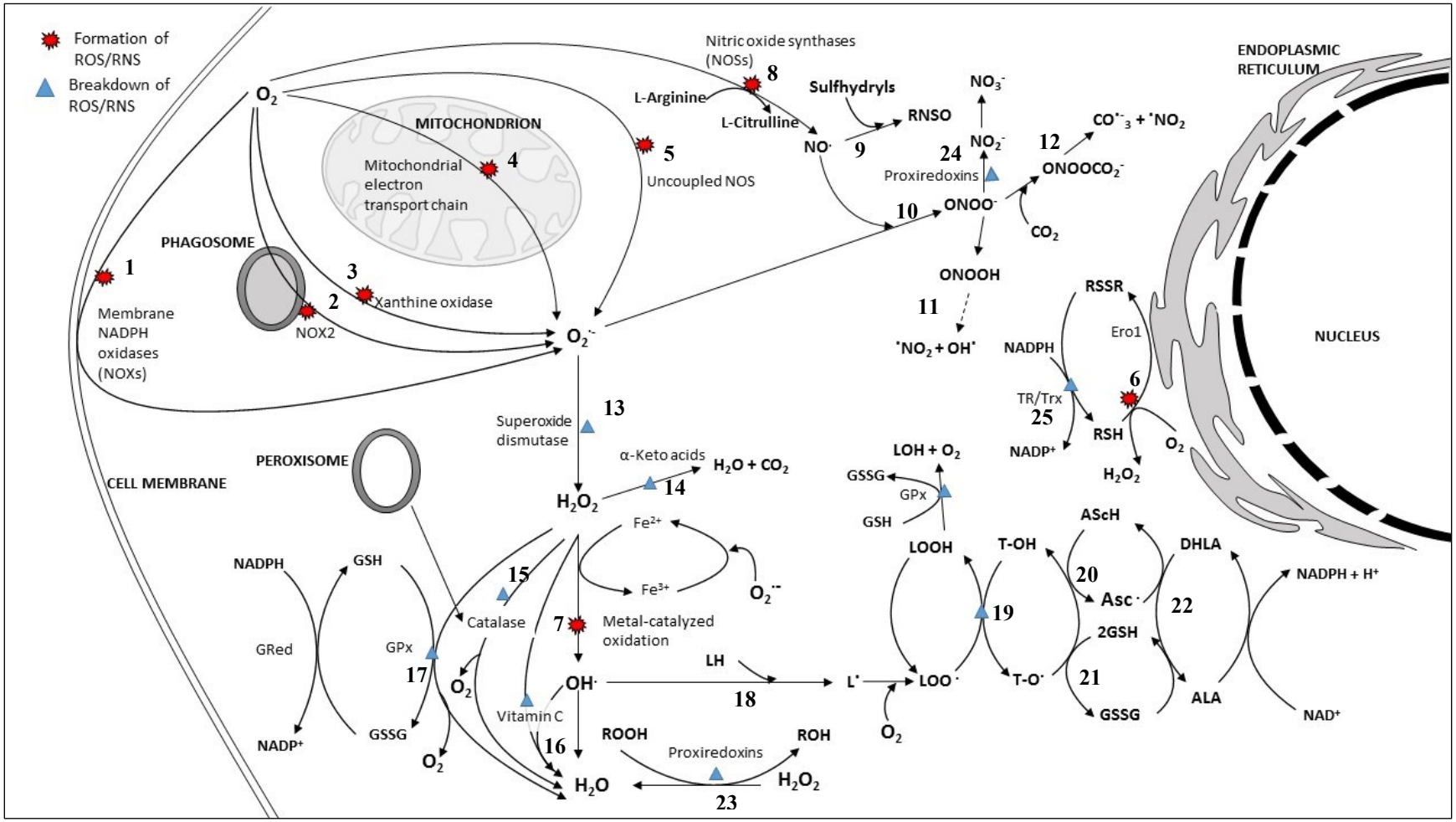
Formation of ROS/RNS
Breakdown of ROS/RNS
MITOCHONDRION
Mitochondrial electron transport chain
PHAGOSOME
Membrane NADPH oxidases (NOXs)
NOX2
Xanthine oxidase
Uncoupled NOS
Nitric oxide synthases (NOSs)
L-Arginine
L-Citrulline
Sulfhydryls
RNSO
Proxiredoxins
ONOO⁻
ONOOH
CO2
PEROXISOME
CELL MEMBRANE
Superoxide dismutase
α-Keto acids
Catalase
Metal-catalyzed oxidation
Vitamin C
Proxiredoxins
ENDOPLASMIC RETICULUM
NUCLEUS
Ero1
RSSR
RSH
NADPH
NADP+
TR/Trx
GSH
GSSG
GPx
GRed
LOOH
LOH + O2
T-OH
T-O·
LOO·
LH
L·
AScH
Asc·
2GSH
GSSG
DHLA
ALA
NADPH + H+
NAD+

Figure 2.1: Formation, reaction and breaking down pathways of ROS and RNS in cell (Gathered from (Nathan & Ding, 2010; Valko *et al*., 2007; Pacher, Beckman & Liaudet, 2007)**)**

Superoxide radical ($O_2^{\bullet-}$) is formed by the process of reduction of molecular oxygen mediated by NADPH oxidases in cell membrane (NOXs, reaction 1), by activating NOX2 in phagosomes (reaction 2), by xanthine oxidases (reaction 3) or by electrons leaked from mitochondrial electron transport chain (reaction 4). Nitric oxide synthase (NOS) requires tetrahydrobiopterin (BH_4) as cofactor to produce $NO^{\bullet}$. Under suboptimal levels of BH_4 or when uncoupled by hypoxia, NOS no longer produces $NO^{\bullet}$, instead generates $O_2^{\bullet-}$ (reaction 5). Ero1 (ER oxidoreductin) is a glycosylated flavoenzyme belong to the family of sulfhydryl oxidases and catalyzes the formation of disulfide bonds in the endoplasmic reticulum (ER). Ero1 oxidizes protein to form disulfide bonds at the expense of molecular oxygen to generate hydrogen peroxide (H_2O_2, Reaction 6). Transition metal ions such as Fe^{2+}, Cu^{+} can catalyze breakdown of H_2O_2 to produce more reactive hydroxyl radical ($OH^{\bullet}$, reaction 7). Nitric oxide ($NO^{\bullet}$) is formed by nitric oxide synthase (NOS) during the conversion of L-arginine to L-citrulline (reaction 8). $NO^{\bullet}$ can react with sulfhydryl groups in proteins to form S-nitrosothiols (RSNO, reaction 9). Reaction of $O_2^{\bullet-}$ with $NO^{\bullet}$ produces peroxynitrite ($ONOO^{-}$, reaction 10) which can then be protonated to peroxynitrous acid (ONOOH). Decomposition of ONOOH generates $OH^{\bullet}$ nitrogen dioxide ($NO^{\bullet}_2$) radicals (reaction 11). Carbon dioxide (CO_2) can rapidly reacts with $ONOO^{-}$ to form unstable nitrosoperoxycarbonate, ($ONOOCO_2^{-}$), which is then homolyzed to more toxic carbonate radical ($CO_2^{\bullet}$) and $NO^{\bullet}_2$ (reaction 12). Superoxide dismutase (SOD) in the cytosol converts $O^{\bullet-}$ to H_2O_2, (reaction 13). The H_2O_2 may convert to water and CO_2 by α-ketoacids, such as pyruvate, α-ketoglutarate and oxaloacetate etc through oxidative decarboxylation (reaction 14), or to water and molecular oxygen by enzyme catalase (reaction 15). Vitamin C (ascorbate) also reduces H_2O_2 and scavenges $OH^{\bullet}$ (reaction 16). Glutathione peroxidase (GPx) scavenges H_2O_2 using GSH, which donates an electron to form oxidized glutathione (GSSG, reaction 17). GSH regenerates using NADPH as the electron donor mediated by the enzyme glutathione reductase (GRed). Abstracting an electron from polyunsaturated fatty acid by $OH^{\bullet}$ produces lipid radical ($L^{\bullet}$, reaction 18), which further reacts with molecular oxygen to form lipid peroxyl radical ($LOO^{\bullet}$). Vitamin E (T-OH) reduces the $LOO^{\bullet}$ to form vitamin E radical ($T\text{-}O^{\bullet}$, reaction 19). $T\text{-}O^{\bullet}$ can be regenerated by vitamin C ($AScH^{-}$, reaction 20) which results in ascorbyl radical ($ASc^{\bullet}$) or by glutathione (GSH, reaction 21). Both GSH and $AScH^{-}$ can be regenerated by dihydrolipoic acid (DHLA) which is converted into α-lipoic acid (ALA, reaction 22). DHLA can be regenerated by NADPH. Peroxiredoxins possess reactive cysteine, which they use to reduce organice peroxides (ROOH) to their corresponding alcohol (ROH) and H_2O_2 to water (reaction 23) and $ONOO^{-}$ to less reactive nitrite (NO_2^{-}) and nitrate (NO_3, reaction 24). The thioredoxin (Trx) redox cycle is mediated by NADPH and includes the Trx reductase (TR), which reduces the protein disulfides (reaction 25).

2.2. Oxidative Stress, Disease and Aging

In general, the cellular production of ROS/RNS is strictly regulated via a series of enzymatic and non-enzymatic ROS-scavenging systems. The enzymatic antioxidant system consists of an array of well-known enzymes such as superoxide dismutases, catalases and the enzymes of the glutathione redox cycle (Finkel, 2011). Some smaller molecules such as pyruvate, and ascorbate can react with ROS and capable of recycling, thus possessing a ROS-buffering capacity (Nathan & Cunningham-Bussel, 2013). The body's antioxidant defense system is not completely effective and as a result free radical-damaged molecules are continually formed and accumulated (Halliwell, 1994; Sies, 1997). The presence of various repair enzymes in the system could destroy damaged proteins and oxidized lipids, repair DNA damages, and control the levels of unincorporated irons (Davies, 2000; Imlay, 2008; Kryston, Georgiev, Pissis, & Georgakilas, 2011). Some of the oxidative damages are irreversible and subjected to degradation/removal by proteasomes or autophagosomes (Scherz-Shouval & Elazar, 2011). Failures in repairing or removing these damaged macromolecules can contribute to disease pathology including carcinogenesis (Gupta *et al*., 2012; Waris & Ahsan, 2006), neurodegeneration (Federico *et al*., 2012; M. T. Lin & Beal, 2006), atherosclerosis (Kaneto, Katakami, Matsuhisa, & Matsuoka, 2010; Sugamura & Keaney, 2011), diabetes (Kaneto *et al*., 2010), and aging (Liochev, 2013).

Excessive formation of ROS in cells, which developed as a result of decreased cellular defense capacity and/or increased ROS generation, can create a stress condition, commonly referred to as oxidative stress. Oxidative stress results in direct or indirect ROS-mediated damage of nucleic acids, proteins, and lipids etc (Halliwell, 1994; Sies, 1997). Not only ROS cause pathogenesis of diseases via macromolecular damage, but alterations in redox signaling is implied as an

additional contributor (Finkel, 2011). Initiating and promoting cancers and neurodegenerative diseases are perhaps the most well-known pathological consequences of ROS. ROS mediated DNA damages, including DNA base modifications, mainly the transversion of guanine to thymine (G to T), rearrangement and miscoding in sequences, and DNA lesions are viewed as the major factors in carcinogenesis (Reuter, Gupta, Chaturvedi, & Aggarwal, 2010). Abnormal signalling of ROS associated with oncogenes or tumor suppressor genes can result in tumor initiation/progression (Waris & Ahsan, 2006). Reaction of lipid peroxidation products such as malondialdehyde (MDA) with DNA leads to the formation of numerous DNA adducts, i.e., 8-hydroxydeoxyguanosine (8-OHdG or 8-oxodG), a well-characterized and most widely used biomarker of oxidative DNA damage (Ménézo, Dale, & Cohen, 2010). It has been shown that primary breast tumors accumulate 8- to 17-fold higher contents of 8-OHdG compared to that of normal breast tissue (Malins & Haimanot, 1991). Under oxygen deprived conditions (hypoxia), cells produce transcriptional factors including HIF-1 (hypoxia inducible factor-1) to up-regulate certain genes and to promote cell survival. Accumulation of HIF-1 is positively correlate with increased levels of H_2O_2 and $O_2^{\bullet -}$, contributing to tumour progression, angiogenesis and chemoresistance (Reuter *et al*., 2010). In general, increased ROS levels and significantly altered antioxidant defense systems can be observed in cancer cells, indicating their adaptability in survival and growth. However, at an advanced stage, the cells exhibit genetic instability and produce a large amounts of ROS (Trachootham, Alexandre, & Huang, 2009).

The human brain is one of the most metabolically active organs with a comparatively lower capacity of cellular regeneration and believed to be more susceptible to the harmful effects of ROS (Shukla, Mishra, & Pant, 2011; Waris & Ahsan, 2006). ROS damages in specific regions in brain are linked to many neurodegenerative diseases such as Alzheimer's disease (AD),

Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). In AD, the patients experience a progressive memory loss as a result of extensive neuronal damage. ROS-mediated amyloid β-protein damage and a higher level of lipid peroxidase were observed in brain cortex and hippocampus of AD patients (Waris & Ahsan, 2006). ALS is characterized by progressive degeneration of motor neurones. It has been suggested that mutation in the gene of Cu/Zn-superoxide dismutase (SOD-1) is a major causative factor of ALS (Shukla *et al*., 2011). Increased level of lipid peroxidases in spinal fluids and accumulation of damaged proteins, lipids, and DNA were observed in ALS patients (Andersen, 2004). Parkinson's disease is also a neurodegenerative movement disorder. Increased levels of lipid hydroperoxides, significantly lower reduced-glutathione (GSH) content in the *substantia nigra* region and accumulation of oxidized proteins in Lewy bodies (distinctive neuronal inclusions found in PD) were observed in patients with Parkinson disease (Valko *et al*., 2007).

There are multiple and complex roles of ROS in various cardiovascular pathological conditions such as atherosclerosis, ischemic heart disease, hypertension, stroke etc. Among many reactive species, $O_2^{\bullet-}$, H_2O_2 and $NO^{\bullet}$ are of particular importance in the cardiovascular system (Al Ghouleh *et al*., 2011). Increased levels of ROS in vascular walls cause direct cellular damage by triggering a series of redox sensitive signalling pathways, altering the cell towards a pro-atherogenic environment (De Marchi, Baldassari, Bononi, Wieckowski, & Pinton, 2013). Up-taking of oxidized low density lipoproteins (ox-LDL) by macrophages via scavenger receptor leads to accumulation of foam cells, the hallmark of early atherosclerotic lesions (Sugamura & Keaney, 2011). Additionally, foam cells can release pro-inflammatory cytokines and growth factors altering the local inflammatory responses. Oxidized LDL is known to increase the production of ROS in endothelial cells and vascular smooth muscles, inducing the expression of

adhesion molecules on endothelial cells, proliferation of macrophages and activation of platelets (Goyal *et al*., 2012). ROS generated from Nox and mitochondria shown to modify the composition of atherosclerotic lesions and alter the expression of mitochondrial antioxidants (De Marchi *et al*., 2013). Regulation of mitochondrial expression on antioxidants is of vital importance as it is shown that both over-expression (Loor *et al*., 2011) and deficiency (Y. Li *et al*., 1995) of antioxidants such as manganese superoxide dismutase (MnSOD) can induce myocardial injuries. ROS/RNS are known to involve in pathophysiology of hypertension, a leading cause of cardiovascular diseases, via increased vascular cell proliferation and migration, apoptosis, inflammation and extracellular matrix alterations (Schulz, Gori, & Münzel, 2011). The peroxynitrile radical formed by reacting $O_2^{\bullet-}$ with $NO^{\bullet}$, can uncouple the endothelial nitric oxide synthase (eNOS) causing it to produce ROS rather than nitric oxide reducing its bioavailability (Schulz *et al*., 2011).

The mitochondrial free radical theory of aging (MFRTA), first proposed by Harman implies that the aging is caused by ROS toxicity which is caused by ROS-induced mitochondrial damage leading to further generation of ROS (Harman, 1972; Hekimi, Lapointe, & Wen, 2011). The maximum lifespan and the rate of aging in vertebrates including mammals and birds are shown to correlate with mitrochondrial ROS production and degree of fatty acid unsaturation in tissues (Barja, 2013). Over-expression of human peroxisomal catalase in transgenic mice showed to increase their lifespan by 17–21% compared to their wild-type counterparts, where the catalases are believed to suppress the levels of mitochondrial ROS, agreeing with MFRTA (Schriner, Linford, & Martin, 2005). However, except for the above catalase example, most cellular antioxidants have no associations with aging process (Perez-Campo, López-Torres, Cadenas, Rojas, & Barja, 1998). Although increased oxidative stress was observed in nematode *C.*

elegans lacking mitochondrial SOD-2, this did not shorten their lifespan as expected, instead prolonged it which directly opposed the MFRTA theory (Van Raamsdonk & Hekimi, 2009). In general, it is proposed that, while ROS is closely linked to aging process, it is not the initial nor the indispensable causing factor of aging (Hekimi *et al*., 2011).

2.3. Antioxidants in Human Health

An antioxidant can be defined as ‘‘any substance that delays, prevents or removes oxidative damage to a target molecule’’(Halliwell, 2007) or ‘‘any substance that directly scavenges ROS or indirectly acts to up-regulate antioxidant defences or inhibit ROS production’’ (Khlebnikov, Schepetkin, Domina, Kirpotina, & Quinn, 2007). The human body produces many enzymatic and nonenzymatic endogenous antioxidants in order to provide the primary defense against superoxide and hydrogen peroxides. The major antioxidant enzymes are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GRx) and peroxiredoxins (Carocho & Ferreira, 2013). Nonenzymatic endogenous antioxidants are coenzyme Q10, vitamin A, glutathione, uric acid, lipoic acid, bilirubin, L-carnitine etc (Carocho & Ferreira, 2013; Rizzo *et al*., 2010). There are many different mechanisms by which antioxidants exert protective effects against oxidative damage. They can scavenge free radicals and other reactive species by stopping initiation or propagation of free radicals chain reactions in the system, scavenging singlet oxygen, sequestering transition metal ions to prevent generation of free radicals, reducing localized oxygen concentration, and inhibiting pro-oxidative enzymes such as lipoxygenases (Brewer, 2011; Carocho & Ferreira, 2013; Lobo, Patil, Phatak, & Chandra, 2010). Antioxidants can work synergistically with each other against different types of free radicals and reactive species. The most efficient enzymatic antioxidants are glutathione

peroxidase (GSH-Px), catalase, and SOD (MatÉs, Pérez-Gómez, & De Castro, 1999). GSH-Px and SOD (in two forms: CuZnSOD and MnSOD) are found in mitochondria and cytosol, whereas catalases are located in peroxisomes (Rodriguez *et al*., 2004). SOD converts superoxide into H_2O_2 and oxygen, while GSH-Px and catalase react with H_2O_2 to produce water and oxygen (Rodriguez *et al*., 2004). Although the gene expression and activity of these enzymes in the cell are well regulated to maintain redox homeostasis, internal and external factors such as aging, inflammation, smoking and toxins can influence the balance (MatÉs *et al*., 1999).

Glutathione (GSH) is a water soluble tripeptide (L-γ-glutamyl-L-cysteinylglycine) that can react with ROS using its thiol group and oxidized to form glutathione disulfide (GSSG) which can then convert back to GSH by combined action of NADPH cofactor and GRx (Carocho & Ferreira, 2013; Rizzo *et al*., 2010). GSH is also involved in regeneration of ascorbate (Carocho & Ferreira, 2013). Coenzyme Q10, present in all cells and membranes, is the only endogenously synthesized liposoluble antioxidant. It is an effective antioxidant which prevents lipid peroxidation during the initiation step and is involved in regenerating vitamin E (Littarru & Tiano, 2007). Uric acid is a metabolic product of purine nucleotide, which is absorbed back into the body during kidney filtration into the plasma (Carocho & Ferreira, 2013). It is a potent singlet oxygen and hydroxyl radical scavenger and prevents lysis of red blood cells by peroxidation (Ames, Cathcart, Schwiers, & Hochstein, 1981).

2.3.1. Dietary Antioxidants

Intake of antioxidants through diet is thought to be important in reducing the oxidative damage (Halliwell, 1996; (Halliwell, 2012; Sies, 1997; Valko *et al*., 2007). These antioxidants play a critical role in protecting cellular components from potentially damaging ROS and thereby

maintaining homeostasis and optimal cellular functions. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and propyl gallate (PG) have been used in both food and pharmacological applications (Gülçin, 2012). However, because of the possible toxic and carcinogenic effects associated with BHT and BHA, their use is legally restricted (Vandghanooni *et al*., 2013; Williams, Iatropoulos, & Whysner, 1999). As a result, there is a growing interest in using natural antioxidants for food and therapeutic applications which prompt the scientific community to explore new sources of natural and dietary antioxidants (Carocho & Ferreira, 2013; Gülçin, 2012; Halliwell, Murcia, Chirico, & Aruoma, 1995; Samaranayaka & Li-Chan, 2011). The most well-known groups of natural antioxidants are vitamin C, vitamin E, carotenoids and flavonoids and more recently, peptides with antioxidant properties derived from various plant and animal sources (Gülçin, 2012; Samaranayaka & Li-Chan, 2011). Most of the plant derived antioxidant compounds are phytochemicals including phenolics, flavonoids and carotenoids whereas the prominent animal-derived antioxidants are amino compounds such as amino acids, peptides and proteins (Sikora, Cieślik, & Topolska, 2008).

Vitamin E, a well-known chain breaking antioxidant, prevents propagation of lipid peroxidation reactions by donating its phenolic hydrogen to the lipid peroxyl radical (Gülçin, 2012). Vitamin E will become a radical itself (tocopheroxyl radical), but is more stable due to delocalization of the solitary electron over the aromatic ring structure (Gülçin, 2012; Niki, 2014). Lipid soluble vitamin E is considered the most important antioxidant in preventing lipid peroxidation. Carotenoids are another class of lipid soluble compounds with antioxidant properties. The main mechanisms are singlet oxygen quenching, reacting with free radicals and delocalizing the unpaired electrons with the aid of unsaturation and resonant stabilization (Stahl & Sies, 2003;

Tang, 2013). Singlet oxygen scavenging ability of lutein and zeaxanthin is suggested as the main protective mechanism of eye macular against blue light-induced oxidative damage (Alves-Rodrigues & Shao, 2004; Ma & Lin, 2010). Carotenoids can also prevent lipid peroxidation and play a protective role in carcinogenesis (Zhang, Cooney, & Bertram, 1991). Although beneficial at moderate concentration, high doses of supplementation of β-carotenoids in high concentration can act as a pro-oxidant (Stahl & Sies, 2003; Procházková *et al*., 2011).

Vitamin C or ascorbic acid, a water soluble vitamin, has been shown to be effective against the superoxide radical anion, H_2O_2, the hydroxyl radical and singlet oxygen (Benzie & Choi, 2014; Gülçin, 2012). It also acts synergistically with vitamin E by reacting with tocopheroxyl radical to regenerate its antioxidant ability (Fang, Yang, & Wu, 2002). Flavonoids represent a class of phytochemicals which are known to have antioxidant properties depending on structural features such as the number and position of the hydroxyl groups and number of phenolic rings etc (Procházková, Boušová, & Wilhelmová, 2011; Rice-Evans, Miller, & Paganga, 1996). They have been reported to scavenge peroxyl radicals, inhibit lipid peroxidation, and chelate metal ions (Brunetti, Di Ferdinando, Fini, Pollastri, & Tattini, 2013; Procházková *et al*., 2011).

Fruits, vegetables, oil seeds, nuts, cereals, spices, herbs, and grains are important sources of antioxidants such as phenolics, flavonoids and carotenoids. A great deal of research has been conducted on their antioxidant properties *in vivo, in vitro* as well as on extraction and purification methods, applications in food products, bioavailability, and anti-nutritional aspects (Abourashed, 2013; Duthie & Crozier, 2000; Pokorný, 1991; Re *et al*., 1999; Rice-Evans, Miller, Bolwell, Bramley, & Pridham, 2009). Among many plant sources, berries and fruits are known for their high phenolic content including phenolic acids, and anthocyanins and their high antioxidant capacity (Kahkonen *et al*., 1999). Fruits such as bilberry, crowberry, cranberry and

apples contain high amounts of phenolic acids, flavonoids, anthocyanins, polycyanidins and also vitamin C, which may contribute towards their antioxidant capacity (Kahkonen *et al*., 1999; Sikora *et al*., 2008). Most of the common vegetables including tomatoes, red pepper, *Brassica* vegetables, onion, garlic and red beet are found to have high antioxidant capacity mainly attributed to their flavonoid, carotenoid, vitamin C contents (Cao, Sofic, & Prior, 1996; Chu, Sun, Wu, & Liu, 2002; Podsędek, 2007; Velioglu, Mazza, Gao, & Oomah, 1998). Although cereal grains are not considered as rich sources of antioxidants compared to fruits and vegetables, grains and grain products are staple food components in the human diet and therefore their contribution is still significant (Adom & Liu, 2002; Dykes & Rooney, 2007; Van Hung, 2014). The major phenolic compounds are phenolic acids such as ferulic acid, the dominating phenolic acid in wheat, caffeic acid, *p*-coumaric acid, p-hydroxybenzoic acid, vanillic acid and protocatechuic acid etc (Van Hung, 2014). In addition, they contain other compounds which may exert antioxidant effects, for example, vitamin E, folates, minerals (iron, zinc) and trace elements (selenium, copper and manganese), carotenoids etc. It has been suggested that antioxidant capacity of cereals is usually underestimated because of the bound phenolic compounds which do not contribute during *in vitro* assays, but can be released in the gut to exert the antioxidant activity (Fardet, Rock, & Rémésy, 2008; Pérez-Jiménez & Saura-Calixto, 2005).

Compared to antioxidants from plant sources, the available research on animal-derived antioxidants is limited. Proteins and peptides have been known to inhibit lipid oxidation through inactivation of reactive oxygen species, scavenging free radicals, chelation of prooxidative transition metals, reduction of hydroperoxides, and alteration of the physical properties of food systems (Elias, Kellerby, & Decker, 2008). The most abundant antioxidant dipeptides in skeletal muscles are histidine-containing dipeptides, such as carnosine and anserine

(Chan & Decker, 1994). The peptide concentration varies from about 500 mg per kg of chicken thigh to 2700 mg per kg of pork shoulder depending on the type of muscle (Elias, Kellerby, & Decker, 2008). Their antioxidant properties are believed to arise through radical scavenging and metal chelation abilities (Chan & Decker, 1994). The presence of thiol groups and aromatic side chains (tryptophan, tyrosine and phenyl alanine) and imidazole ring in histidine (Atmaca, 2004; Stadtman & Levine, 2003) are recognized as important structural features for their potent antioxidant properties. Casein derived peptides from milk proteins have been reported to inhibit enzymatic as well as non-enzymatic oxidation of lipids (Sakanaka, Tachibana, Ishihara, & Juneja, 2005; Suetsuna, Ukeda, & Ochi, 2000). Generation of antioxidative peptides from milk proteins has been studied in detail (Power, Jakeman, & FitzGerald, 2013). Antioxidant peptides from egg proteins have also been reported (Dávalos, Miguel, Bartolomé, & López-Fandiño, 2004; Huang, Majumder, & Wu, 2010). Apart from proteins, other antioxidant compounds in animal tissues such as vitamin E and ascorbic acid are well-known for their antioxidant properties (Sies & Stahl, 1995). Some aquatic animals including salmon and shrimp contain high amounts of carotenoids with strong antioxidant properties. Astaxanthin, a carotenoid found in high concentrations in fish and shrimp, showed strong singlet oxygen and radical scavenging ability, which was 100 times greater than α-tocopherol activity (Miki, 1991). The activity was mainly attributable to the presence of hydroxyl and keto endings on each ionone ring in the structure of astaxanthin (Ngo, Wijesekara, & Vo, 2011).

2.3.2. *In vitro* and *in vivo* measurements of antioxidants

In vitro chemical assays provide simple and rapid ways to assess antioxidant capacity of a wide range of compounds (Kohen & Nyska, 2002; Sanchez-Moreno, 2002). These assays usually

provide the basic information on antioxidant capacity of the compound interested. The many types of available antioxidant assays to date have both advantages and disadvantages and extensively evaluated for their biological relevance, simplicity, mechanisms, quantitation methods (Karadag, Ozcelik, & Saner, 2009; Kohen & Nyska, 2002; Niki, 2010; Sanchez-Moreno, 2002). Based on the chemistry involved, the major *in vitro* antioxidant assays can be categorized as (1) assays using hydrogen atom transfer (HAT) reactions, and (2) assays using electron transfer (ET) reactions (Huang, Ou, & Prior, 2005). The most common *in vitro* chemical assays used to measure antioxidant activities of food and biological materials are total phenolic contents assay by Folin−Ciocalteu reagent (TPC), oxygen radical absorbance capacity assay (ORAC), diphenyl-1-picrylhydrazyl copper (II) reduction capacity (DPPH), Trolox equivalent antioxidant capacity (TEAC) and the ferric reducing antioxidant power (FRAP) assay. Assays such as, TRAP (total radical trapping antioxidant parameter) and ORAC use HAT mechanism and others such as TPC, DPPH and ABTS use ET mechanism during the assay reaction. In general, it is suggested that TPC is used to quantify an antioxidant's reducing capacity and the ORAC assay to quantify peroxyl radical scavenging capacity (Huang et al., 2005). In addition to these, other specific assays are needed to comprehensively study different antioxidants (Carocho & Ferreira, 2013; Prior et al., 2003; Sanchez-Moreno, 2002).

In vivo measurements provide biological relevant information on efficacy and activity of an antioxidant. Effects of antioxidants have been studied by analysis of levels of oxidative biomarkers such as oxidation products of lipids and proteins, modifications in DNA and oxidation products of DNA bases in biological fluids and tissues, such as plasma, erythrocytes, urine, and cerebrospinal fluids, from humans and experimental animals (Niki, 2010). A recent study reported that 3 weeks of oral supplementation of *Cynara scolymus* (artichoke) leaf extract

in the streptozotocin-induced diabetic rat model resulted in decrease in serum malondialdehyde and 8-hydroxydeoxyguanosine (8-OHdG) levels, whereas erythrocyte glutathione levels significantly increased (Magielse *et al*., 2014). A human clinical study designed to find the antioxidant effects of fruit preparation from *Lycium barbarum* (goji) which involved 50 healthy adults, showed the serum levels of endogenous antioxidants SOD and GSH-Px significantly increased by 8.4% and 9.9% between the preintervention and postintervention measurements, whereas MDA were significantly decreased by 8.7%. There were no effects found in the placebo group (Amagase, Sun, & Borek, 2009).

In general, some *in vivo* antioxidant measurements confirmed the positive effects of antioxidant compounds to reduce the level of oxidative stress status while some studies did not show any significant effect. It is also suggested that antioxidants may be more efficient in reducing oxidative stress biomarkers under disease or stress conditions, than under normal healthy conditions (Niki, 2010).

2.4. Egg as an Antioxidant Food Commodity

2.4.1. Egg Industry: Current Market and Consumer Trends

Eggs contain all necessary nutrients required to sustain a new life and have been a popular food consumed by humans for centuries. Adverse public perception on the association between egg cholesterol and the risk of cardiovascular diseases had a devastating impact on the egg industry in the developed world especially in the 1980s; in Canada, the egg production decreased from 480 million of dozens in 1988 to 465 million of dozens in 1990, mainly driven by the media coverage on the association of egg and cholesterol (Hailu & Goddard, 2004). However, it is now understood that egg consumption is not associated with increased risk of cardiovascular diseases

(Ballesteros *et al*., 2014; Nakamura *et al*., 2007; Qureshi *et al*., 2007; Rong *et al*., 2013; Shin, Xun, Nakamura, & He, 2013). With the popularization of low-carb Atkin's diet and emerging of specialty eggs, such as free range/organic eggs and nutrient enriched eggs, table egg sales started to increase (Bejaei, Wiseman, & Cheng, 2011). During the past decade, from 2003 to 2013, egg production in Canada has increased from 584 million dozens to 664 million dozens, while retail egg sales in 2013 gained a 3.4% growth compared to the previous year (Statistics Canada 2013; Egg Farmers of Canada 2013). Globally, egg production has increased rapidly in the recent decades driven mainly by increasing protein requirement in the developing world; in 2012, the world egg production reached 71.9 million metric tons, compared to 57.7 million metric tons in 2002 (FAOSTAT 2014). China is the leading egg producer contributing to over 40% of the total production, followed by USA, Japan and India with 7.5%, 5% and 3.5% of shares respectively, of the total production (FAOSTAT 2014).

Although cholesterol reduced eggs did not have much success (Elkin, 2007a, 2007b; Hargis, 1988), 'specialty eggs' including 'enriched eggs' or 'designer eggs' have gained increased momentum. The term 'specialty eggs' has been used to identify free-range eggs, organic eggs, nutrient enriched eggs etc (Bejaei *et al*., 2011). Goddard and colleagues conducted research across two Canadian provinces, Alberta and Ontario, aimed to assess the factors influence consumers preferences on different types eggs (Goddard, Boxall, Emunu, & Boyd, 2007). It was revealed that in both provinces, families with children tend to buy free run and organic eggs, while older people are more willing to buy omega 3 and vitamin enriched eggs (Goddard *et al*., 2007). A survey conducted in British Columbia, Canada in 2009, showed that 32.9% of the participants consumed free-range eggs and 11.9% consumed organic eggs compared to the 8% (all types of specialty eggs from the total participants) in 2007 (Bejaei *et al*., 2011). Similarly, in

the United Kingdom, the market sales of free-range chickens in 2013 were 50% of the total table egg sale (British Egg Industry Council, 2014). In summary, the consumers are more concerned on the nutritional value, price as well as animal welfare while purchasing eggs.

2.4.2. Chemical and Nutritional Composition of Eggs

Egg is composed of three parts: egg shell with membranes, egg white albumen, and yolk, accounting for approximately 9.5%, 63% and 27.5% of the whole shell egg (Cotterill & Geiger, 1977). The edible portion of the egg consists of water (74%), proteins (12%), lipids (12%), carbohydrate (<1%) as well as vitamins and minerals (Li-Chan *et al.*, 1995). The chemical and nutrient composition of egg is shown in Table 2.1. The protein fraction is distributed in both egg white (ovalbumin, ovotransferrin, ovomucoid, ovomucin etc) and yolk (high density lipoproteins, low density lipoproteins and livetins). Egg proteins are high quality proteins and are used as a golden standard for measuring the quality of other food proteins (Seuss-Baum, 2007). Almost all egg lipids are located in yolk and approximately 65% of yolk lipids are triglycerides, while phospholipids, cholesterol and carotenoids make 30%, 4%, <1%, respectively (Hatta, Kapoor, & Juneja, 2008). The fatty acid composition of egg yolk can be manipulated through feed formulation to produce eggs enriched with polyunsaturated fatty acids with benefits beyond basic nutrition (Surai *et al.,* 2000). Based on the composition of standardized poultry feed, about 30–35% from the total fatty acids are saturated fatty acids (SFA), 40–45% are monounsaturated fatty acids (MUFA), and 20–25% are polyunsaturated fatty acids (PUFA) (Anton, 2007). Egg yolk lipids have been used as a source of long-chain polyunsaturated fatty acids, DHA and phospholipids to incorporate into infant formula (Simopoulos and Salem, 1992; Carlson *et al.,* 1996, Hoffman *et al.,* 2004). Eggs are also considered a good source of micronutrients such as vitamins and minerals. Eggs contain ~16, 29, 9 and 9% of the recommended daily intake (RDI)

of phosphorus, selenium, iron, and zinc, and 10% of the RDI of vitamin A, D, E, K, B2, B12, biotin and pantothenic acid (Seuss-Baum, 2007). It has been shown that some minerals like selenium, and iodine can be enriched through fortification of feed (Bourre & Galea, 2006; Charoensiriwatana, Srijantr, Teeyapant, & Wongvilairattana, 2010). In the same way as minerals, vitamin contents of egg can be manipulated through hen's feed formulation (Naber, 1993).

Table 2.1: Nutrient composition of whole egg, egg yolk and egg white (in 100 g, without shell)

Nutrients	Egg white	Egg yolk	Whole egg
Energy content (kcal)	47	364	154
Water (g)	88.6	49	74.4
Protein (g)[a]	10.6	16.1	12.3
Carbohydrate (g)	0.8	0.5	0.7
Ash (g)	0.5	1.6	0.9
Fat (g)	0.03-0.1	34.5	11.9
Triglycerides (g)		22.9	7.7
Phospholipids (g)		10.0	3.4
Cholesterol		1.2	0.42
Lecithin		7.2	2.3
Saturated fatty acids (g)	-	13.0	4.4
16:0 palmitoleic acid		7.3	2.5
18:0 Stearic acid		2.5	0.86
Unsaturated fatty acids (g)		20.7	7.0

16:1 palmitic acid		1.1	0.4
18:1 oleic acid		12	4.1
18:2 linoleic acid		3.6	1.25
18:3 linolenic acid (n-3)		0.12	0.04
20:4 arachidonic acid (n-6)		0.6	0.2
20:5 EPA (n-3)		0	0
22:6 DHA (n-3)		0.4	0.15
Essential amino acids (mg)			
Histidine[b]	290	416	309
Isoleucine	240	410	290
Leucine	560	870	660
Lysine	880	1390	1040
Methionine + Cystine	660	1170	820
Phenylalanine + Tyrosine	670	660	640
Threonine	1020	1420	1150
Tryptophan	470	850	590
Valine	170	240	190
Vitamins (µg)			
Ascorbic acid	0	0	0
Vitamin A, Retinol eq.	0	450	150
Vitamin D	0	4.5	1.5
Vitamin E	0	3600	1200
Vitamin B_1	10	250	91.3
Vitamin B_2	430	480	447
Vitamin B_6	10	370	133

Vitamin B_{12}	0.1	2.8	1-2
Folate	12	140	56
Niacin	90	60	79
Biotin	7	60	25
Pantothenic acid	250	4500	1700
Minerals (mg)			
Sodium	155	50	120
Chlorine	175	162	172
Potassium	140	100	125
Calcium	8	133	50
Phosphorous	15	390	198
Iron	0.1	4.8	1.7
Magnesium	10	15	12
Sulphur	163	165	164
Zinc	0.12	3.9	1.4
Copper	0.02	0.14	0.06
Manganese	0.007	0.11	0.04
Iodine	0.003	0.14	0.05

[a] Table information gathered from (Kovacs-Nolan, Phillips, & Mine, 2005; Li-Chan, 2008; Seuss-Baum, 2007; USDA, 2014)

In addition to the nutritional value, egg components have various biological activities which may render important health benefits. Egg is a complete biological system designed to nourish and protect the growing embryo from various pathogen invasions. As a result, egg shell with membranes and egg white proteins possess physical and biological defense mechanisms such as

viscosity, pH, antimicrobial properties etc. Egg compounds with various bioactivities are listed in Table 2.2.

Table 2.2 Major egg components with biological activities [a]

Egg component	Biological activity
Egg white proteins	
Ovalbumin	Anticancer activity
	Highly antimutagenic against pepper-induced mutagenicity (Hosono, Shashikanth, & Otani, 2009)
	Antihypertensive activity of peptides from ovalbumin (Fujita, Sasaki, & Yoshikawa, 1995; Matoba, Usui, Fujita, & Yoshikawa, 1999; Miguel, Aleixandre, Ramos, & López-Fandiño, 2006)
	Antimicrobial activity of peptides from trypsin digested ovalbumin (Pellegrini & Hülsmeier, 2004)
	Antioxidant activity: Restricts lipid oxidation (Huang, Tu, Xiao, Wang, & Zhang, 2012; Nakamura, Kato, & Kobayashi, 1992)
Ovotransferrin	Antimicrobial activity: Bateriostatic and bactericidal activity, antifungal and antivital activities, iron-chelating antimicrobial properties (Giansanti *et al*., 2005; H. R. Ibrahim, Sugimoto, & Aoki, 2000; Valenti *et al*., 1983; Valenti, Visca, Antonini, & Orsi, 1985)
	Immunomodulating: Modulates macrophages and heterophil function (Xie, Huff, Huff, Balog, & Rath, 2002), inhibits

	proliferation of mouse spleen lymphocytes (Otani & Odashima, 1997)
	Antioxidant activity: SOD-like superoxide scavenging activity due to metal chelating ability (Ibrahim, Hoq, & Aoki, 2007)
Ovomucoid	Protease inhibitor: Inhibits trypsin and chymotrypsin (Fraenkel-Conrat, Bean, & Lineweaver, 1949; Lineweaver & Murray, 1947)
	Immunomodulating activity: secretion of cytokines upon induced by a synthetic peptide (Holen, Bolann, & Elsayed, 2001)
	Biospecific ligand: Prevent degradation of protein drugs (Agarwal, 2000)
Ovomucin	Antiadhesive activity: Binds *E. coli* and protects glycopeptides (Kobayashi *et al*., 2004)
	Antitumor activity (Oguro, Ohaki, Asano, Ebina, & Watanabe, 2001)
	Antimicrobial activity (Shan *et al*., 2013)
	Antioxidant activity: Inhibit H_2O_2-induced oxidative stress in human embryonic kidney (Chang, Ha, & Han, 2013)
Lysozyme	Antimicrobial activity: Inhibit *E.coli and S. aureus* (Hughey & Johnson, 1987)
	Antitumor activity: Inhibit tumor growth after oral administration (Sava, Benetti, Ceschia, & Pacor, 1988; Sava, Ceschia, & Zabucchi, 1988)
	Antioxidant activity: Suppress ROS and oxidative stress genes (Liu *et al*., 2006)

	Immunomodulating: Enhances IgM production (Murakami, Sasaki, & Sugahara, 1997)
Ovoinhibitor	Serine protease inhibition (Shechter, Burstein, & Gertler, 1977; Tomimatsu, Clary, & Bartulovich, 1966)
Ovomacroglobulin	Protease inhibition: protease inhibitory activity towards trypsin, papain, and thermolysin (Kitamoto, Nakashima, & Atsushi, 1982)
Cystatin	Cysteine protease inhibitor (Nicklin & Barrett, 1984)
	Antimicrobial activity: Bactericidal effect on *E. coli* (Wesierska *et al*., 2005)
	Antioxidant activity (Verdot *et al*., 1996)
	Anticancer activity: Suppress tumor growth (Keppler, 2006)
Avidin	Anticancer activity: Use in cancer treatments to localize and image cancer cells (Corti *et al*., 1998; Yao *et al*., 1998)
	Antimicrobial activity: Inhibit microbes through biotin binding (Elo, Räisänen, & Tuohimaa, 1980; Korpela, Salonen, Kuusela, Sarvas, & Vaheri, 1984)
	Biospecific ligand: Facilitates delivery of therapeutics through blood brain barrier (Bickel, Yoshikawa, & Pardridge, 2001)

Egg yolk components

Immunoglobulin Y (IgY)	Antimicrobial activity: IgY is functionally equivalent to human

	IgG. Inhibits *E. coli, Salmonella* spp., *Yersinia* spp., and *Helicobacter pylori* etc (Yoshinori Mine & Kovacs-Nolan, 2002; Sunwoo, Lee, Menninen, Suresh, & Sim, 2002)
Phosvitin	Antibacterial activity: Inhibits *E. coli* (Sattar Khan *et al*., 2000)
	Antioxidant activity based on metal chelating ability, also (Lu & Baker, 1986; Young, Nau, Pasco, & Mine, 2011)
Sialyloligosaccharides and Sialyloligopeptides	Antiadhesive properties
Yolk lipids	Antibacterial activity: Inhibits *Streptococcus* spp. (Brady *et al*., 2003)
Lipoproteins	Antibacterial activity: Anti-Streptococcal activity (Brady, Gaines, Fenelon, Mcpartlin, & O'Farrelly, 2002)
Fatty acids	Antibacterial activity: Triglycerides inhibit the growth of *Streptococcus* spp. (Brady *et al*., 2003)
Phospholipids	Antioxidant activity: Inhibit lipid oxidation (Sugino *et al*., 1997)
	Role in brain development an function: (Carlson, Montalto, & Ponder, 1998)
	Cholesterol lowering activity: Cholesterol absorption was suppressed by phosphatidylcholine (Homan & Hamelehle, 1998) and by sphingomyelin (Eckhardt, Wang, Donovan, & Carey, 2002) in Caco-2 cells and by phospholipids in rats (Murata, Imaizumi, & Sugano, 1982)
Cholesterol	Essential component of cell membranes (Yeagle, 1985)

[a] Table information gathered from (Kovacs-Nolan *et al*., 2005; Y Mine & D'Silva, 2007)

2.4.3. Antioxidant Compounds in Eggs

Numerous compounds in both egg white and yolk exhibit antioxidant properties. Many egg proteins such as ovalbumin, ovotransferrin, phosvitin, egg lipids such as phospholipids, as well as certain micronutrients such as vitamin E, vitamin A, selenium, and carotenoids, are reported to have antioxidant properties. In addition, eggs can be further enriched with antioxidants (i.e. carotenoids, vitamin E, selenium and iodine) through manipulation of poultry feed (Chung, Rasmussen, & Johnson, 2004; Dobrzanski, Gorecka, Strzelbicka, Szczypel, & Trziszka, 2001; Surai & Sparks, 2001; Surai, 2000).

2.4.4. Antioxidants Naturally Occurring in Eggs

2.4.4.1. Ovalbumin

Ovalbumin is a glycoprotein made of 385 amino acids and constitutes approximately 54% (w/w) of the total egg white protein (Lechevalier & Croguennec, 2007; Li-Chan, 2008). It contains six cysteine residues with a single disulfide bond and is the only egg white protein with free SH (thiol) groups (Li-Chan, 2008). The presence of thiol groups enable its ability to play a role in redox regulation and binding metal ions therefore exert antioxidant properties (Deneke, 2001; Roos & Messens, 2011; Thomas, Poland, & Honzatko, 1995). In 1971, Goto and Shibasaki observed the protective effects of ovalbumin against lipid oxidation in a linolenic model system (Goto & Shibasaki, 1971). When covalently attached with polysaccharides, the radical scavenging activity of ovalbumin was significantly increased (Nakamura, Kato, & Kobayashi, 1992). It was speculated that free SH groups are responsible for the antioxidant activity of ovalbumin, which were effectively exposed upon the conjugation with polysaccharides (Nakamura *et al., 1*992). Further studies on glycated ovalbumin showed that the activity is

dependent on the type of sugars used and also the configuration of hydroxyl groups in the sugar molecule (Huang *et al.*, 2012; Sun & Hayakawa, 2006).

2.4.4.2. Ovotransferrin

Ovotransferrin (also known as conalbumin), representing 12-13% of the total egg white protein, is a member of the transferrin family, a group of ion-binding proteins with an *in vivo* preference for iron (Li-Chan, 2008; Superti & Ammendolia, 2007). Ovotransferrin consists of two lobes, each capable of binding one atom of Fe^{3+} and carbonate anion (Li-Chan, 2008). Among the two, the N-lobe is found to be more important for its antioxidant properties (Ibrahim *et al.,* 2007). Ovotransferrin was reported to possess superoxide dismutase (SOD)-like activity against superoxide anion promoted by metal binding. The scavenging activity was dose-dependent and considerably higher than known for antioxidants such as ascorbate or serum albumin (H. Ibrahim *et al*., 2007). Additionally, the iron-binding ability of ovotransferrin has an indirect role in preventing iron-induced lipid peroxidation (Li-Chan and Nakai, 1989).

2.4.4.3. Lysozyme

Lysozyme is an enzyme present in almost all organisms. One egg contains approximately 0.3-0.4 g of lysozyme (Mine & Kovacs-Nolan, 2004). Lysozyme is a defensin, a member of the family of native, highly conserved host-defense proteins (Liu *et al.*, 2006). It contains an 18-amino acid domain that binds agents such as advanced glycation end products (AGE), which contribute to the production of reactive oxygen species and increased oxidative stress (ROS). Liu *et al*., (2006) showed that lysozyme protects transgenic mice against acute and chronic oxidative injury. They also showed that hepatocytes incubated with lysozyme suppress cellular ROS levels and

oxidative response genes. In another study, the survival rate following acute or chronic oxidative injury in lysozyme deficient transgenic mice was found to be significantly lower compared to the control, indicating its protective role as an antioxidant (Fritz & Ikegami, 2009).

2.4.4.4. Cystatin

Egg white cystatin is the first identified member of the cystatin family (Fossum & Whitaker, 1968). It is a small protein of approximately 13 kDa molecular weight which makes up 0.05% of the total egg white proteins and contains two disulfide bonds (Li-Chan, 2008). Cystatin is an inhibitor of cysteine proteinases, thereby exerting antibacterial properties (Nicklin & Barrett, 1984; Wesierska *et al.,* 2005). It is also reported that chicken cystatin exerts immunomodulatory activities by modulating the synthesis and release of $NO^{\bullet}$ production in interferon γ-activated murine macrophages (Verdot *et al.,* 1996; Verdot *et al.,* 1999; Vray, Hartmann, & Hoebeke, 2002). It is known that optimum levels of $NO^{\bullet}$ is essential for regulation of certain cellular antioxidant pathways (Abbas *et al.,* 2011). Moreover, cystatin B, the group which chicken cystatin belongs to, has recently found to involve in protecting cerebellar granule neurons from oxidative stress by playing a role in oxidative stress-responsive signalling pathway (Lehtinen *et al*., 2009). Taken together, the role of cystatin in modulating the $NO^{\bullet}$ synthesis and protecting brain neurons from oxidative damage, provide us with evidence of its potential activity as an antioxidant.

2.4.4.5. Ovoinhibitor

Ovoinhibitor, which makes approximately 1.5% of egg white proteins, inhibits serine proteinases such as trypsin and chymotrypsin and also bacterial and fungal proteinases (Yoshinori Mine &

Kovacs-Nolan, 2004). It was shown that chymotrypsin proteinase inhibitors including ovoinhibtor are capable of inhibiting the formation of ROS in activated human polymorphonuclear leukocytes during the inflammatory response (Frenkel, Chrzan, Ryan, Wiesner, & Troll, 1987). They demonstrated that about 29% of formation of H_2O_2 was inhibited by ovoinhibitor at a concentration of 20 μM (Frenkel *et al.*, 1987).

2.4.4.6. Phosvitin

Phosvitin is the most phosphorylated protein containing nearly 80% of yolk protein phosphorous and represents ~11% of yolk proteins (Joubert & Cook, 2011). More than half of its amino acid composition is serine, which exists as phosphoserine. It has a strong metal-binding ability and approximately 95% of yolk iron is bound to phosvitin. This high metal-binding capacity makes phosvitin a potential antioxidant, particularly against iron induced oxidative damage (Lu & Baker, 1986). Iron is essential for life; under normal physiological conditions, the level is controlled by iron binding proteins ferritin and transferrin. However, if the balance is disturbed causing iron overload in cells, the effects could be lethal as humans have a very limited capacity to excrete excess iron. The excess iron in the form of Fe^{2+} can participate in Fenton reaction to produce toxic $OH^{\bullet}$ by reacting with H_2O_2 (reaction IV).

$$Fe^{2+} + H_2O_2 \rightarrow OH^- + \bullet OH + Fe^{3+} \quad \text{(IV)}$$

Moreover, the circulating free iron can oxidize heart-muscle membranes, causing arrhythmia and heart failure (Nathan, 1995). The iron-chelating ability of phosvitin indicates its possible role in protecting iron-induced oxidative damage. Phosvitin accelerates Fe^{2+} autoxidation, thereby reducing the availability of Fe^{2+} and inhibiting Fe^{2+}-catalyzed $OH^{\bullet}$ generation through Fenton

reaction (Ishikawa *et al*. 2004). Additionally, phosvitin is also proven to be effective against UV-induced lipid peroxidation in the presence of excess iron (Ishikawa *et al.* 2005).

2.4.4.7. Phospholipids

Egg yolk phospholipids consist of 84% phosphatidylcholine (PC), 12% phosphatidylethanolamine (PE), 2% sphingomyelin and 2% lysophosphatidylcholine and other minor compounds (Hatta *et al.*, 2008). King, Boyd, & Sheldon (1992), reported that egg yolk phospholipids exhibit antioxidant activity in a refined salmon oil model system, and also demonstrated that the presence of nitrogen improved the antioxidant activity of phospholipids. The antioxidant activity was positively associated with the degree of fatty acid unsaturation (Sugino *et al.,* 1997). Hydroxy amines in the side chains of choline and ethanolamines showed strong inhibition of lipid peroxidation, indicating the importance of side-chain amino acids with hydroxyl groups in the antioxidant activity (Saito & Ishihara, 1997).

2.4.4.8. Carotenoids

Carotenoids are lipid soluble compounds responsible for the orange-yellow color of the egg yolk. The health promoting properties of carotenoids have attracted many researchers and are well documented (Rao, 2007). More than 600 carotenoids have been identified to date and it is suggested than around 50 of them might occur in our diet and 14 in human blood (Rao & Rao, 2007; Voutilainen, Nurmi, Mursu, & Rissanen, 2006). Bioavailability of egg carotenoids is superior to those from green leafy vegetables (Chung *et al*., 2004; Handelman *et al*., 1999) due to the solubilization of yolk lipids, which makes eggs a unique and important carrier of bioactive carotenoids. The profile of egg carotenoids is largely depend on hen's feed composition,

therefore it can vary among different types of eggs (Karadas, Grammenidis, Surai, Acamovic, & Sparks, 2006; Schlatterer & Breithaupt, 2006). Certain carotenoids are allowed to use as poultry feed additives to improve color of the egg yolk, however, the amount and types of carotenoid can be varied as per the country's feed regulation (Breithaupt, 2007). In general, lutein, zeaxanthin, canthaxanthin, β-apo-8'-carotenal, capsanthin, β-apo-8'-carotenoic acid ethyl ester, β-cryptoxanthin, and citranaxanthin can be present in egg yolk (Breithaupt, 2008).

Human plasma contains several carotenoids including β-carotene, α-carotene, β-cryptoxanthin, lutein and zeaxanthin and their isomers (Krinsky & Russett, 1990). Lutein, zeaxanthin and *meso*-zeaxanthin are the main components of the eye macular pigment (Landrum & Bone, 2001). Lutein and zeaxanthin are well known for their role in protecting the eye from age-related macular degeneration (AMD) (Krinsky, Landrum, & Bone, 2003). The singlet oxygen and radical scavenging activity of lutein and zeaxanthin is considered one of the two major mechanisms for their beneficial effects against light-induced oxidative damage in eye macular, in particular, against AMD (Böhm, Edge, & Truscott, 2012; Krinsky *et al.*, 2003; Li, Ahmed, & Bernstein, 2010). The other major mechanism is their ability to absorb blue light, particularly before it damages the photoreceptor cells, which is also considered a passive antioxidant action (Krinsky *et al*., 2003). A recent study demonstrated that pre-incubation of human lens epithelial cells (HLEC) with lutein, zeaxanthin and α-tocopherol, dramatically reduced the levels of H_2O_2 -induced protein carbonyl, MDA, and DNA damage (Gao *et al*., 2011). Further, lutein, zeaxanthin and α-tocopherol supplementation increased GSH levels and GSH:GSSG ratio, particularly in response to oxidative stress (Gao *et al*., 2011). Dietary supplementation with lutein reduced plasma lipid hydroperoxides and the size of aortic lesions in mice (Dwyer *et al.,* 2001) and reduced the plasma levels of oxidized-LDL in guinea pigs (Kim *et al.,* 2011), indicating a

protective role in ROS induced early atherosclerosis. The ability of lutein and zeaxanthin to scavenge hydroxyl and superoxide radicals is attributed to the presence of double bonds which makes a bond with the free radical to produce a highly resonance-stabilized C-centered radical (Trevithick-Sutton, Foote, Collins, & Trevithick, 2006). Lutein, zeaxanthin and β-cryptoxanthin have also been shown to scavenge peroxynitrite which may play a role in LDL protection against oxidative damage (Panasenko, Sharov, Briviba, & Sies, 2000).

2.4.4.9. Vitamins and Minerals

On average egg contains around 1.1 mg of vitamin E (Seuss-Baum, 2007) which is equivalent to 8.5% of RDA. Vitamin E, especially α-tocopherol as the most active form, is a well-known lipophilic chain-breaking antioxidant known to protect long-chain polyunsaturated fatty acids in the membranes of cells and thus maintain their bioactivity (Burton & Traber, 1990; Traber & Atkinson, 2007). In plasma, vitamin E exists with LDL and HDL, providing protection against oxidation (Esterbauer *et al.,* 1991; Ricciarelli *et al.,* 2000). Supplementing with vitamin E increased resistance to LDL-oxidation and is associated with a lower risk of coronary diseases in both men (Rimm *et al.,* 1993) and women (Stampfer *et al.,* 1993). Eggs can be enriched with vitamin E to provide up to 150% RDA without formation of off flavour (Surai *et al.,* 2000), not only providing the aforementioned benefits, but also protecting against oxidation of long chain fatty acids in yolk (Surai *et al.,* 2000).

Certain minerals present in egg yolk including selenium and iodine also contribute to the antioxidant properties. Selenium is an essential mineral present in antioxidant selenoproteins such as glutathione peroxidases (GPx), thioredoxin reductases (TrxR) and selenoprotein P (Sepp1) (Tapiero, Townsend, & Tew, 2003). Iodine has a potential role as an antioxidant in

human systems including the eye, thyroid and the breast (Smyth, 2003). Iodine deficiency can increase the stimulation of thyroid gland by TSH resulting in excessive H_2O_2 (Smyth, 2003).

2.4.4.10. Egg-derived Antioxidative Peptides

Antioxidant activity was reported from egg white and egg yolk proteins. Recently, many studies have reported antioxidant properties of egg white proteins hydrolyzed using different enzymes and some have even purified the potential antioxidant peptides (Chen, Chi, & Xu, 2012; Chen Chen, Chi, Zhao, & Lv, 2012; Lin *et al.,* 2013; Remanan & Wu, 2014). Trypsin hydrolysate prepared from egg white protein precipitate, obtained as a by-product in cystatin and lysozyme isolation, showed a considerably better radical scavenging activity than those prepared from chymotrypsin and elastase (Graszkiewicz, Zelazko, & Trziszka, 2010; Graszkiewicz & Zelazko, 2007). Adult male spontaneously hypertensive rats fed with peptic digested egg white for 17 weeks showed increased radical-scavenging capacity of the plasma and lowered MDA concentration in the aorta, and exerted a beneficial effect on the lipid profile, lowering triglycerides and total cholesterol without changing HDL levels (Manso *et al*., 2008). Two peptides derived from lecithin-free egg yolk exhibit protection against lipid peroxidation in intoxicated normal human liver cells (Park, Jung, Nam, Shahidi, & Kim, 2001). Both peptides contained a leucine residue at their N-terminal positions which was thought to contribute to their antioxidant properties (Park *et al*., 2001). Another study showed that egg yolk protein hydrolysate exhibited superoxide and hydroxyl radical scavenging activity, effectively inhibiting thiobarbituric acid reactive substances (TBARS) formation from ground beef and tuna homogenates, indicating its potential as a natural antioxidant (Sakanaka & Tachibana, 2006).

The peptide, Tyr-Ala-Glu-Glu-Arg-Tyr-Pro-Ile-Leu, derived from pepsin hydrolyzed ovalbumin, which was previously reported to possess angiotensin converting enzyme (ACE)-inhibitory activity, also exhibited a strong radical scavenging activity and delayed the LDL-oxidation induced by Cu^{2+} (Dávalos *et al*., 2004). Peptic digests of ovalbumin inhibited the action of $OH^{\bullet}$ and $O_2^{\bullet-}$ and also prevented the oxidation of linoleic acid in linoleic acid autoxidation system (Xu, Shangguan, Wang, & Chen, 2007). *In-vivo* studies showed that supplementation with these peptic digests of ovalbumin significantly decreased the production of oxidants and oxidative damage in serum and liver of aged mice (Xu *et al*., 2007).

Enzymatic hydrolysis of ovotransferrin was shown to lead to enhanced overall antioxidant activity. Two tetrapeptides (Trp-Asn-Ile-Pro and Gly-Trp-Asn-Ile) were characterized from thermolytic hydrolysate of ovotransferrin (Shen, Chahal, & Majumder, 2010). Trp-Asn-Ile was suggested as the responsible peptide motif for the high activity of the above tetrapeptides (Huang *et al*., 2012). A tripeptide Ile-Arg-Trp, derived from ovotransferrin showed strong radical scavenging activity which was attributed to tryptophan and the peptide bond between Trp and Arg (Huang *et al*., 2012). It is known that Trp can exert radical scavenging properties mainly due to the presence of the indole ring (Christen, Peterhans, & Stocker, 1990; Galisteo & Herraiz, 2004). A recent study demonstrated that grafting a catechin moiety significantly increased the antioxidant activity of ovotransferrin implicating its potential as neutraceutical and functional food (You, Luo, & Wu, 2014). Peptides derived from lysozyme are reported to possess antioxidant properties (Memarpoor-Yazdi, 2012; Rao *et al*., 2012; You, Udenigwe, Aluko, & Wu, 2010).

Egg white ovomucin, a sulfated glycoprotein accounts for 3.5-4% of egg white proteins, is responsible for the jelly-like structure of egg white (Li-Chan, 2008; Omana, Wang, & Wu,

2010). Recently, ovomucin derived pentapeptide Trp-Asn-Trp-Ala-Asp was reported to reduce H_2O_2-induced oxidative stress in human embryonic kidney (HEK-293) cells by inhibiting intracellular ROS accumulation and blocking the ROS activated mitochondria-mediated cell apoptosis pathway (J. Liu *et al*., 2014). Others also reported on antioxidant properties of peptides derived from ovomucin (Chang *et al*., 2013; Remanan & Wu, 2014).

Phosvitin phosphopeptides (PPP) obtained from tryptic digestion of egg yolk phosvitin showed protective effects against H_2O_2-induced oxidative stress in human intestinal epithelial cells (Katayama, Xu, Fan, & Mine, 2006; X. Xu, Katayama, & Mine, 2007). The antioxidative activity of PPP was similar to that of glutathione and positively related to the phosphorous content. PPPs are also assumed to be involved with up-regulating glutathione and associated antioxidative enzymes such as glutathione reductase, glutathione *S*-transferase, and catalase and thus reducing the oxidative stress (Katayama & Mine, 2007). Furthermore, the antioxidative activity of PPPs on H_2O_2-induced oxidative stress was retained after gastrointestinal digestion (Young *et al*., 2011).

2.4.5. Enriched Antioxidants in Egg

Owing to its high lipid content, many lipid-soluble antioxidant compounds such as lutein/zeaxanthin, vitamin E, selenium, iodine lycopene can be incorporated into egg yolk (Surai, Simons, Dvorska, Aradas, F., & Sparks, 2006). The most studied are omega-3 fatty acids, which are incorporated into eggs by feeding fish oil, flax seed, algae, or other ingredients to laying hens (Fraeye *et al*., 2012). High contents of omega-3 fatty acids might increase susceptibility to fatty acid oxidation therefore simultaneously enrichment of eggs with antioxidants such as vitamin E

and carotenoids was suggested to decrease fatty acid oxidation and provide a good source of dietary antioxidant (Surai *et al*., 2006).

Carotenoids are naturally occurring in egg yolk in varied amounts depending on hen's feed. Feed fortification with natural sources such as marigold (*Tagetes erecta*) or alfalfa (*Medicago sativa*) extracts provide lutein, while other sources such as corn (*Zea mays*) and red pepper (*Capsicum annuum*) provide zeaxanthin and capsanthin respectively (Breithaupt, 2007; Leeson, 2006). Canthaxanthin, β-apo-8'-carotenal and β-apo-8'-carotenoic acid ethyl ester are chemically synthesized and incorporated into the feed (Breithaupt, 2008). Lutein and zeaxanthin are two major egg carotenoids that can be found in human serum, skin and eye macular and involved in the protective roles against oxidative stress (Roberts, Green, & Lewis, 2009; Serpeloni, Grotto, Mercadante, de Lourdes Pires Bianchi, & Antunes, 2010). Lutein content of enriched eggs can be increased up to 15-fold compared to the control group, for example enriched egg contains around 1.9 mg of lutein (Surai, MacPherson, Speake, & Sparks, 2000). Lutein enriched eggs show a higher lutein bioavailability compared to lutein, lutein ester supplements, and spinach (Chung *et al*., 2004). Lycopene is a hydrocarbon carotenoid reported to have strong antioxidant properties effective in reducing the risk of prostate carcinoma (Wertz *et al*., 2004; Olson, Ward, & Koutsos, 2008). Although lycopene is not usually found in eggs, lycopene enrichment can be achieved via feed fortification with tomato powder and lycopene could reduce yolk lipid peroxidation (Akdemir, Orhan, Sahin, Sahin, & Hayirli, 2012).

Vitamin E is the major lipophilic antioxidant compound in our body that may provide the primary protection against free radical induced lipid peroxidation (Traber & Atkinson, 2007). The daily requirement is approximately 15 mg α-tocopherol equivalents per day (Péter & Moser,

2013). Since vitamin E is needed to protect membrane lipids from being peroxidized, this amount can be increased with higher intake of polyunsaturated fatty acids (Sanders & Hinds, 2007; Valk & Hornstra, 2000). Egg can be enriched to provide around 20 mg of vitamin E per egg, which is more than the daily requirement, and also provide protection against unsaturated fatty acid peroxidation (Surai *et al*., 2006). Folate, a water soluble B-group vitamin is shown to reduce the incidence of neural tube defects in newborns (Honein, 2001). Egg yolk can be enriched with highly bioavailable folate through fortification of feed with folic acid to provide up to 12.5% of the recommended daily intake of folate (House, Braun, Ballance, O'Connor, & Guenter, 2002; House, O'Connor, & Guenter, 2003). Almost all the folate in egg exists in the form of 5-methyltetrahydrofolate (5-MTHF), and showed high stability during cooking (Seyoum & Selhub, 1998). Folates are reported to have antioxidant properties and among different forms, 5-MTHF was reported to have the most prominent antioxidant activity, which was attributed to the electron donating effect of the 5-amino group (Rezk, Haenen, van der Vijgh, & Bast, 2003). *In vivo* and *ex vivo* studies with human vessels showed that 5-MTHF improves $NO^{\cdot}$-mediated endothelial function, decreases superoxide production, scavenge peroxynitrite and also reversed eNOS uncoupling, thereby exerts antioxidant effects (Antoniades *et al*., 2006; Verhaar *et al*., 1998).

Both selenium and iodine, which are known to have antioxidant properties, can be effectively transferred into the egg yolk. Eggs can be supplemented to provide up to 50% and 150% of the daily requirements of selenium and iodine respectively (Dobrzanski *et al*., 2001; Surai *et al*., 2006). Collectively, these antioxidant enriched eggs provide multiple advantages by serving as a dietary source of several nutrients including omega 3, vitamin E, vitamin D, selenium, iodine and also as an important source of antioxidants such as lutein.

2.5. Effect of Processing, Storage Conditions and Gastrointestinal Digestion on Food Antioxidants

Foods are subjected to various processing and storage conditions before consumption, which may influence the antioxidant capacity of food components. The effect of food processing and storage conditions on the overall antioxidant activity of a particular food is a result of several different events occurring consecutively or simultaneously (Nicoli, Anese, & Parpinel, 1999). According to Nicoli *et al*., (1999), there are three possible effects of food processing on the overall antioxidant capacity:

1. The total antioxidant capacity was not affected: as a result of no changes in natural antioxidant compounds or loss of naturally occurring antioxidants balanced by formation of compounds with novel or improved antioxidant properties,
2. The total antioxidant capacity was increased: as a result of improvement of antioxidant properties of naturally occurring compounds or formation of new antioxidant,
3. The total antioxidant capacity was decreased: loss of naturally occurring antioxidants or formation of new compounds with pro-oxidant activity.

Most thermal processing can cause oxidation, thermal degradation, and leaching of vitamin C and phenolic compounds, which would reduce the antioxidant activity (Kalt, 2005). With regard to carotenoids, processing can lead to the dissociation of compounds from plant matrix resulting in increased carotenoid antioxidants, and improved digestive absorption (Dewanto, Wu, Adom, & Liu, 2002; Dewanto, Wu, & Liu, 2002; Shi & Le Maguer, 2000). Most of the fruits and vegetables contain phenolic compounds, carotenoids and vitamin C, which are differently affected by processing conditions. Consequently, the total antioxidant capacity can be increased (Adefegha & Oboh, 2013; Ravichandran *et al*., 2013; Turkmen, Sari, & Velioglu, 2005) or

decreased (Ismail, Marjan, & Foong, 2004; Natella, Belelli, Ramberti, & Scaccini, 2010; Ravichandran *et al*., 2013; Zhang & Hamauzu, 2004).

In animal-derived foods, the antioxidant capacity depends mainly on amino compounds (proteins, peptides and amino acids) and vitamin E. An early study revealed that heating could expose sulfhydryl groups from cysteine, leading to increased activity (Taylor & Richardson, 1980). The antioxidant activity of human breast milk was significantly decreased at both refrigeration and freezing storage and freezing resulted in a greater decrease (Hanna *et al*., 2004). The decrease may be attributed to loss of milk antioxidants, uric acid, α- and γ-tocopherols, carotenoids, vitamin A and vitamin C, and enzymes such as catalase and glutathione peroxidase and free amino acids like tryptophan (Hanna *et al*., 2004; Tsopmo *et al*., 2009). Serpen, Gökmen, & Fogliano (2012) reported that the antioxidant capacity of beef, chicken, pork, and fish increased during the first 5 min of cooking but decreased afterwards until 15 min, and then slightly increased at the final stage of heating. These changes were attributed to exposure of reactive protein sites after protein denaturation, degradation of endogenous antioxidants and formation of Maillard reaction products with antioxidant properties (Serpen *et al*., 2012). Jensen, Dort, & Eilertsen (2014) also reported that cooking reduced antioxidant capacity of pork and beef.

Heat modification of egg white proteins, ovalbumin, lysozyme and ovomucoid via Maillard reaction resulted in protein-sugar conjugates, leading to increased radical scavenging properties (Jing, Yap, Wong, & Kitts, 2009). Chen, Chi, & Xu (2011) showed that there are no significant differences in terms of DPPH radical-scavenging activity, reducing power, and lipid peroxidation inhibitory activity of spray dried and freeze-dried egg white protein hydrolysates compared to the undried sample. Antioxidant properties of egg yolk phosvitin is due to its iron binding

abilities; heating phosvitin at 110 °C for 40 min did not change the iron binding ability of phosvitin (Albright, Gordon, & Cotterill, 1984).

Carotenoids and vitamin E in egg yolk are reported to be influenced by thermal processing. In the presence of heat, light, oxygen etc, carotenoids can undergo *trans-cis* isomerization, or they can be degraded resulting in altered or loss of bioactivity (Schieber & Carle, 2005). Boiling of eggs resulted in a 10-20% carotenoid loss (Schlatterer & Breithaupt, 2006), whereas pasteurizarion did not change the carotenoid content (Wenzel, Seuss-Baum, & Schlich, 2010). Storage conditions such as temperature can also affect the antioxidant properties of eggs. Storage at refrigeration temperature for two weeks reduced significantly the total carotenoid content in raw eggs enriched with omega-3 and carophyll (canthaxanthin preparation), while at room temperature, the losses were observed after 7 days of storage (Barbosa, Gaspar, Calixto, & Agostinho, 2011). The vitamin E content of eggs was also significantly reduced by thermal processing accompanied with increased lipid oxidation products (Caboni, Boselli, & Messia, 2005; Galobart & Barroeta, 2001).

Gastrointestinal digestion involves extreme pH conditions and various enzymes which might cause degradation of antioxidant componds or generation of novel antioxidant compounds. Many recent research activities have evaluated the changes in antioxidant capacity of different food products after gastrointestinal digestion using diverse model systems. After gastrointestinal digestion, the antioxidant activity of wheat (Mateo Anson, Havenaar, Bast, & Haenen, 2010), gooseberries (Chiang, Kadouh, & Zhou, 2013), grapes (Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010), soymilk (Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2013), saithe and shrimp (Jensen, Abrahamsen, Maehre, & Elvevoll, 2009) and loach protein hydrolysate (L. You, Zhao, Regenstein, & Ren, 2010) increased several times, attributed mainly

to increased free amino acid content and short chain peptides generated during digestion. However, the antioxidant activity of some foods such as apples (Bouayed, Hoffmann, & Bohn, 2011) and *Feijoada* whole meal (a traditional Brazilian dish containing vegetables) (Faller, Fialho, & Liu, 2012) was significantly reduced.

Many studies have reported the formation of antioxidant peptides after simulated gastrointestinal digestion of egg components (S. Rao *et al*., 2012; Remanan & Wu, 2014; Young *et al*., 2011). A recent study showed that different types of domestic cooking methods such as boiling and frying decreased the antioxidant activity (Remanan & Wu, 2014). Nevertheless, simulated gastrointestinal digestion of cooked egg with pepsin and pancreatin significantly increased the antioxidant activity, which was attributed to the release of amino acids and antioxidant peptides (Remanan & Wu, 2014). Lutein and zeaxanthin, the main egg carotenoids, remain stable during the gastrointestinal digestion (Blanquet-Diot, Soufi, Rambeau, Rock, & Alric, 2009), and also highly bioaccessible due to the association with yolk fat (Xavier, Mercadante, Garrido-Fernández, & Pérez-Gálvez, 2014), it is likely that they retain their antioxidant activity.

2.6. Summary

Oxidative stress is hypothesized to be responsible for the onset and development of various diseases and ageing. Dietary antioxidants are thought to impart potential benefits in reducing the risk of some chronic diseases by maintaining redox homeostasis. There is extensive research on the presence and characterization of antioxidants from fruits, vegetables, cereals and herbs; however, there is only limited research with regard to antioxidants from animal products. Eggs are an important part of our breakfast and an excellent source of high quality proteins, lipids, vitamins and minerals. Many egg proteins such as ovalbumin, ovotransferrin, phosvitin, and egg

lipids such as phospholipids, as well as certain micronutrients such as vitamin E, vitamin A, selenium, and carotenoids, are reported to have antioxidant properties. Furthermore, eggs can be enriched with antioxidants (i.e. carotenoids, vitamin E, selenium and iodine) through manipulation of poultry feed. Domestic cooking tended to reduce the antioxidant activity of egg, while gastrointestinal digestion of cooked eggs increased the antioxidant due to the release of amino acid and peptides.

CHAPTER 3 - Free Aromatic Amino Acids in Egg Yolk Show Antioxidant Properties[1]

[1]A version of this chapter has been published: Nimalaratne, C., Lopes-Lutz, D., Schieber, A., & Wu, J. (2011). Free aromatic amino acids in egg yolk show antioxidant properties. *Food Chemistry*, 129(1), 155-161.

CHAPTER 3

3.1 Introduction

Eggs are considered as one of nature's perfect foods that have been consumed for centuries all over the world. Although they contain all the necessary nutrients for a new life, consumption of eggs in many developed countries has declined due to the public perception on its high content of cholesterol. However, current evidence suggests that there is no direct link between egg consumption and blood cholesterol level (Lee & Griffin, 2006; Qureshi *et al*., 2007). Egg yolk is a rich source of both nutritive and non-nutritive compounds important to human health. It is well known that hens' diet influences the yolk composition. Through dietary manipulation, certain phytochemicals with important health benefits can be enriched in the egg yolk (Surai & Sparks, 2001). Over the past few years, many research studies have demonstrated that bioactive feed compounds may be transferred from hens' feed into the yolk. Lutein and zeaxanthin are the most extensively studied phytochemicals in egg yolk. They can help prevent age-related macular degeneration (AMD) and eye cataract either as antioxidants and/or by filtering harmful blue light (Chung, Rasmussen, & Johnson, 2004; Moeller, Jacques, & Blumberg, 2000). Phytoestrogens with potential health benefits such as soy isoflavones have also been reported to be present in egg yolk (Kuhnle *et al*., 2008; Saitoh, Sato, Harada, & Matsuda, 2004).

Phenolic compounds commonly found in cereal grains, fruits and vegetables are important antioxidants that are suggested to play a preventive role in the development of many chronic human diseases such as cancer and cardiovascular diseases (Adom, Sorrells, & Liu, 2005; Li, Pickard, & Beta, 2007; Liu, 2007). Yet, limited information is available about the presence of phenolic compounds in egg yolk. In Canada, wheat and corn are cereal grains commonly used in poultry feed rations; both are considered as rich sources of various phenolic compounds such as

ferulic, *p*-coumaric, vanillic acids etc with high antioxidant activities. It has been reported that dietary sinapic acid (4-hydroxy-3,5-dimethoxycinnamic acid) affect egg quality characteristics and low levels of sinapic acid was detected in egg yolk (Johnson, Dahiya, Olkowski, & Classen, 2008); it was also reported that the deposition of simple phenolic acids, unlike the isoflavonoids, in egg yolk is very low under natural conditions. However, it is not known if other phenolic compounds are present in egg yolk.

This study was designed to investigate the possibility of deposition of phenolic acids in egg yolk and the total antioxidant capacity of yolk extracts. It has been reported in the literature that cooking may change the chemical properties of these compounds and therefore affect their antioxidant activity (Li *et al*., 2007; Xu, Li, Lu, Beta, & Hydamaka, 2009). Thus, the effect of cooking methods on the composition and the antioxidant activities were studied. Interestingly, we identified two aromatic amino acids from yolk phenolic extracts with high antioxidant capacity; therefore, the composition of free amino acids of yolk and the effect of cooking methods on free amino acids were also determined.

3.2. Materials and methods

3.2.1. Materials and Chemicals.

Fresh white shell eggs, corn-fed eggs from Ontario and wheat-fed eggs from Manitoba, both were supplied by Burnbrae Farms Limited Canada. Poultry feed samples used by the above farms were also provided by Burnbrae Farms Limited Canada. Fluorescein disodium and Trolox were obtained from Acros Organics (Morris Plains, NJ). Methanol (HPLC and analytical grades), trichloroacetic acid, ferulic acid, gallic acid, 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium

salt (ABTS), 2,2-di (4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH), amino acid standards, ethanolamine (EA), β-aminobutyric acid (BABA) and *o*-phthaldialdehyde (OPA) were purchased from Sigma–Aldrich (Oakville, ON, Canada).

3.2.2. Cooking of Eggs.

Boiled eggs were prepared by placing whole shell eggs in single layer in a saucepan, with water up to 1-2 inches above the egg layer and boiled for 10 min. After boiling they were placed under running tap water for 5 min, peeled and egg yolks were separated from whites. For microwaving, whole eggs were placed in a microwavable glass bowl and cooked in a household microwave oven (model DMW 113w, Danby Products, Ontario, Canada) for 90 seconds at cooking level of 1100 W and 2450 MHz. Egg yolks were carefully separated from whites. Fried samples were prepared using a frying pan preheated to 205 °C (model SK200TY non-stick frying pan, Black & and Decker Canada Inc., Brockville, ON). Whole eggs were fried for 6 min (3 min each side) and the yolks were separated from whites. Raw egg yolks were used as a control. To prepare raw yolk samples, egg yolks were manually separated from whites and wiped with a filter paper to remove adhered albumins. Thirty eggs were used for preparing each sample,i.e. a total of 120 eggs were used for all four samples. Cooked and raw egg yolks were frozen immediately at -20 °C and then subjected to freeze drying in containers impermeable to light. Freeze-dried egg yolks were ground with a coffee blender to obtain a fine homogenized powder and sieved with a 0.425 mm sieve under dim light conditions. The processed samples were stored in the dark at -20 °C in airtight sealed plastic bags until further analysis. Feed samples were ground, sieved under the same conditions and stored at -20 °C.

3.2.3. Extraction of Phenolic Compounds.

To optimize the extraction conditions, preliminary extractions were done with petroleum ether, ethyl acetate, 50% methanol, 100% methanol and 80% acidified methanol and based on the total phenolic content, 80% methanol (pH 1.5) was selected as the extraction solvent. Approximately 1 g of freeze dried egg yolk sample was extracted with 10 mL of 80% methanol (80:20, vol/vol) adjusted to pH 1.5 with 1 M HCl. The sample was then mixed thoroughly using a vortex mixer (standard vortex mixer model 945404, Fisher Scientific, Edmonton, AB, Canada) for 2 min and centrifuged at 6000 *g* for 10 min at 4 °C. The supernatant was evaporated under vacuum at 35 °C using a rotary evaporator and reconstituted with 1 mL of methanol. All samples were filtered with a 0.22 µm Nylon syringe filter and subjected to further analyses. Extracts from feed samples were also prepared using the same procedure mentioned above. Extractions were performed in triplicate.

3.2.4. Determination of Total Phenolic Content (TPC)

The Folin–Ciocalteu assay was used to determine the total phenolic content (Singleton & Rossi., 1965). For this purpose, 0.1 mL of sample was mixed with 0.5 mL of the Folin–Ciocalteu reagent and 1.5 mL of 20% sodium carbonate (Na_2CO_3) solution. The mixture was made up to 10 mL and allowed to stand at room temperature for 2 h. The absorption was measured at 760 nm using a spectrophotometer (Jasco Corp., Tokyo, Japan). Gallic acid was used to develop standard calibration curves and the results were expressed as µmol of gallic acid equivalents (GAE)/g dry weight. Determinations were performed in duplicate for each sample and the values represent the means.

3.2.5. Oxygen Radical Absorbance Capacity-Fluorescein (ORAC-FL) Assay

The ORAC-FL assay was performed according to the method of Ou, Hampsch-Woodill, & Prior (2001) modified by Dávalos, Miguel, Bartolomé, & López-Fandiño (2004). The reaction was carried out in 96F black, untreated 96-well microplates (Nunc, Roskilde, Denmark) and Trolox (2-16 μM) was used as standard. The final assay mixture containing fluorescein (200 nM), 75 mM phosphate buffer (pH 7.4) and the sample at different concentrations was incubated at 37 °C for 10 min. Then, 50 μL of APPH solution (80 mM) were added to each well using a dispenser and the plate was automatically shaken. The fluorescence was recorded using a fluorometric microplate reader (Fluoroskan Ascent; Thermo Electron Corp, Vantaa, Finland) at 1 min intervals for 60 min at excitation and emission wavelengths of 485 and 538 nm, respectively. All assays were conducted in duplicates and at least three independent tests were done for each sample. A blank assay was carried out which did not contain antioxidants. The area under curve (AUC) was calculated for each sample by integrating the relative fluorescence curve. Regression equations obtained from net value of Trolox was used to calculate the ORAC-FL value for each assay. Final ORAC-FL values were expressed as micromoles of Trolox equivalent (TE) per mg of dried yolk (μmol TE/mg).

3.2.6. Free Radical-Scavenging Activity on $ABTS^{\cdot+}$

The free radical scavenging capacity using the ABTS radical cation ($ABTS^{\cdot+}$) is based on the reduction of $ABTS^{\cdot+}$ by antioxidants present in the sample tested. The assay was conducted according Madhujith, Izydorczyk, & Shahidi (2006) with slight modifications. To generate the $ABTS^{\cdot+}$, the ABTS stock solution (7 mM) and potassium persulfate (2.45 mM) in phosphate buffer saline (PBS, pH 7.4) were allowed to stand for 16 h. For the reaction, 100 μL of the

samples was mixed with 100 µL of diluted $ABTS^{\bullet+}$ (absorbance 0.7 at 734 nm) in a 96-well microplate (Costar #9017, Corning, Inc., NY). The decline in absorbance was measured at 734 nm after 8 min incubation at 37 °C (Spectramax 190, Molecular Devices Corporation, CA). A standard curve was prepared using a series of concentrations of Trolox (0, 2, 4, 8, 12, 16 and 20 µM) with 100 µL of diluted $ABTS^{\bullet+}$ solution. The radical scavenging capacity of the yolk samples was calculated based on the Trolox standard curve and was expressed as µmol of TE/mg of dried yolk.

3.2.7. Free Radical-Scavenging Activity on DPPH

The DPPH radical scavenging activity (RSA) of each extract was determined according to the method of Madhujith, Izydorczyk, & Shahidi (2006) with slight modifications. All assays were duplicated for three independent determinations. The DPPH• (0.2 mM) solution in 95% ethanol was mixed with the same volume of sample solution in a 96-well flat bottom microplate (Costar #9017, Corning, Inc., NY) and incubated at 37 °C for 60 min using a shaking incubator. The absorbance was measured at 517 nm using a microplate reader (Spectramax 190, Molecular Devices Corporation, CA). A standard radical scavenging activity curve was prepared by reacting 100 µL of a series of concentrations of Trolox (0, 2, 4, 8, 12, 16 and 20 µM) with 100 µL of diluted DPPH solution. RSA of yolk samples was calculated on the basis of the Trolox standard curve and expressed as µmol of TE/ mg of dried yolk.

3.2.8. HPLC (High-Performance Liquid Chromatography) Analysis of Phenolic Acids

A Waters 600 HPLC system (Waters, Millford, MA) equipped with a 2702 thermoautosampler, a binary gradient pump and a 2998 photodiode array detector was used. All samples were analyzed using a C_{18} reversed-phase column (Waters xBridge 250 mm × 3 mm, i.d., 5 µM) protected with a C_{18} guard column (20 mm × 3 mm, i.d., 5 µM). Mobile phases were 2% acetic acid in water (A) and acetonitrile (B). The flow rate was 0.5 mL/min and the total run time was 60 min with the following linear gradient according to Kim, Tsao, Yang, & Cui (2006): From 0% to 15 % B in 30 min, from 15% to 50% B in 20 min, from 50% to 100% B in 5 min and from 100% to 0% B in 5 min. Between the injections there was a 20 min equilibration time for reconditioning. The analytes were detected at 280 nm and 320 nm. The injection volume was 20 µL. HPLC analyses were performed in duplicates.

3.2.9. HPLC-DAD-ESI-MS/MS Analysis of Phenolic Acids

An, Agilent Technologies 1200 series HPLC equipment including degasser, binary pump, autosampler, thermostatted column compartment and diode array detector (DAD) (Agilent Technologies, Palo Alto, CA) was connected to an Applied Biosystem MDS SCIEX 4000 Q TRAP LC/MS/MS System (AB Sciex, Concord, ON, Canada) for separation and identification of phenolic compounds. Analyst (version 1.5) software was used for data processing. The separation was performed on a C18 reversed phase column (Waters XBridge 250 mm × 3 mm i.d., 5 µM) and a C18 guard column (20 mm × 3 mm, i.d., 5 µM) at a flow rate of 0.5 mL/min. The solvent system and the gradient program were similar as described above. Mass spectrometric data was acquired using an API 4000 QTRAP® hybrid QqQ-linear ion-trap mass

spectrometer operating on a TurboIonSpray® source in negative ionization mode. Major parameters were set as follows: scan speed of 1000 Da/s; 138 kPa for curtain gas; 600 °C for source temperature; 345 kPa for nebulizing gas; 138 kPa for heating gas (GS2) and −3.500 V for spray voltage. An information-dependent acquisition (IDA) method was used to profile the phenolic compounds. Both Q1 and Q3 were operated unit mass resolution. The spectra were obtained over a scan range from *m/z* 50 to 1300 in 2 s. The IDA threshold was set at 100 cps, above which enhanced product ion (EPI) spectra were collected from the eight most intense peaks. The EPI scan rate was 4000 amu s^{-1}. Collision-induced dissociation spectra were acquired using high purity Nitrogen as the collision gas under collision energy (CE) of -20eV. The other MS parameters used were: declustering potential -70 V, entrance potential -10 V and -7 V for collision exit potential. Identification of compounds was achieved based on the retention time, UV spectrum and the fragmentation pattern compared with the authentic standards. The identification of ferulic and isoferulic acids in egg yolk was achieved using multiple reaction monitoring (MRM) scan experiments that included three transitions: 193.0→133.8 (CE -24eV); 193.0→133.2 (CE -40eV) and 193.0→148.8 (CE -16eV).

3.2.10. Preparative HPLC Analysis and Collection of Fractions

Major components identified from egg yolk were isolated using a C_{18} preparative column (150 mm × 10 mm i.d., 5 μM) with C_{18} guard column. The same HPLC system and HPLC conditions similar to those described above were used with a flow rate of 3 mL/min and an injection volume of 477 μL. The two major compounds (P1 and P2 in Figure 3.1) were collected separately, freeze-dried and used for antioxidant assays.

3.2.11. Extraction of Free Amino Acids in Egg Yolk

Free amino acids were extracted using tricholoroacetic acid (Ohkubo *et al*., 2006). Approximately 1 g of the freeze-dried egg yolk was extracted with 5 mL of 6% trichloroacetic acid by vortex mixing for 2 min. The mixture was centrifuged at 6000 g for 20 min at 4 °C and the supernatant was filtered with 0.22 μm Nylon syringe filter (Mandel Scientific Corp., Guelph, ON, Canada) prior injecting to the HPLC analysis.

3.2.12. HPLC Analysis of Free *Amino Acid*s

HPLC analysis of free amino acids was carried out according to the method described by Sedgwick, Fenton, & Thompson (1991). Ethanolamine (EA) and β-amino-n-butyric acid (BABA) were used as internal standards. Quantification was carried out using standard calibration curves.

3.2.13. Statistical Analysis

All data were analyzed by Analysis of variance (ANOVA) followed by Duncan's multiple range test using Statistical Analysis System Software (SAS version 9.0, SAS Institute, Cary, NC). Significance of differences was defined at the 5% level ($p < 0.05$).

3.3. Results and discussion

3.3.1. Total Phenolic Content in Egg Yolk

In this study, four extraction methods were used to determine the optimum conditions of extraction of phenolic compounds from egg yolk. The best extraction conditions were established by analyzing the TPC of the sample and by performing a recovery test with the addition of a ferulic acid standard. Acidified 80% methanol gave the highest TPC and the best

recovery and was thus used for further studies. The total phenolic contents of the yolk samples, expressed as mg of GAE/ 100 g yolk are shown in the Table 3.1. Raw egg yolks showed a TPC content of 72.5 ± 1.1 mg of GAE/ 100 g yolk and 66.1 ± 0.5 mg of GAE/ 100 g yolk in corn-fed and wheat-fed yolks, respectively. Cooking of eggs caused a reduction of the TPC by 5% to 18.3% in both yolk types. The decrease in TPC in fried corn-fed egg yolks was higher than those observed for other cooking methods, whereas in wheat-fed yolks there were no differences among the methods of cooking. It was reported that the degree of polyphenol degradation depends very much on the nature of the food and condition of processing (Sikora, Cieślik, Leszczyńska, Filipiak-Florkiewicz, & Pisulewski, 2008). There were no reports in the literature on the TPC of chicken eggs; however, some studies showed reduced TPC with thermal processing of selected vegetables (Faller & Fialho, 2009; Zhang & Hamauzu, 2004). Boiling was found to be the most damaging cooking method for total phenolics in coloured peppers, whereas stir-frying and microwaving did not cause a significant change (Chuah *et al*., 2008).

Table 3.1 Effect of different cooking methods on total phenolic content (TPC) of wheat-fed yolk samples and corn-fed yolk samples [n]

Cooking condition	Wheat-based yolk		Corn-based yolk	
	TPC (mg GAE /100 g dried yolk)	Loss %	TPC (mg GAE /100 g dried yolk)	Loss %
Raw Yolk	$66.1^{ab} \pm 0.5$		$72.5^{a} \pm 1.1$	
Micro waved	$62.8^{bc} \pm 0.9$	5.0	$66.9^{ab} \pm 7.1$	7.7
Boiled	$58.1^{c} \pm 1.8$	12.1	$67.5^{ab} \pm 2.4$	6.9
Fried	$56.2^{c} \pm 0.2$	15.0	$59.2^{c} \pm 1.0$	18.3

[n] Mean ± SD values in each column with no common superscripts differ significantly ($P < 0.05$)

3.3.2. Antioxidant Properties of Raw and Cooked Yolk Samples

The ORAC-FL assay has been widely applied to measure total antioxidant capacity of many compounds, food and other biological samples. It is the only method that considers both the degree of inhibition and inhibition time in one assay (Prior *et al*., 2003). The ORAC-FL, DPPH and ABTS values obtained in this study are shown in Table 3.2.

Table 3.2 Antioxidant activities of yolk extracts as affected by the method of cooking [n]

	ABTS (µmol TE/ g dry yolk)	DPPH (µmol TE/ g dry yolk)	ORAC (µmol TE/ g dry yolk)
	Wheat-fed yolk		
Micro waved	45.0 ^{e} ± 1.6	6.3 ^{e} ± 0.7	64.5 ^{b} ± 0.3
Boiled	45.4 ^{e} ± 0.5	7.9 ^{de} ± 0.1	77.3 ^{b} ± 4.5
Fried	40.6 ^{e} ± 1.2	12.0 ^{c} ± 0.3	66.3 ^{b} ± 2.2
Raw	54.0 ^{d} ± 0.6	13.2 ^{c} ± 0.2	151.1 ^{a} ± 26.1
	Corn-fed yolk		
Micro waved	55.4 ^{c} ± 0.6	8.6 ^{d} ± 0.9	68.9 ^{b} ± 4.4
Boiled	58.7 ^{b} ± 0.2	13.7 ^{c} ± 0.9	77.9 ^{b} ± 10.7
Fried	64.7 ^{a} ± 0.4	21.3 ^{b} ± 1.9	90.7 ^{b} ± 11.7
Raw	66.0 ^{a} ± 0.9	26.8 ^{a} ± 0.9	155.9 ^{a} ± 9.5

[n] Mean ± SD values in each column with no common superscripts differ significantly ($P < 0.05$)

ORAC-FL values were 151.1 µmol TE/ g dry yolk and 155.9 µmol TE/ g dry yolk in wheat-fed and corn-fed raw yolk samples (Table 3.2), which is comparable to the ORAC values of most

fruits and vegetables listed in the most recently published USDA Database for the Oxygen Radical Absorbance Capacity (ORAC) of Selected Foods, Release 2 (Nutrient Data Laboratory, 2010). Our results showed that cooked eggs have significantly lower ORAC-FL values than those of raw samples. However, there was no significant difference among the boiled, fried and microwaved samples.

Free radical scavenging properties were determined using the $ABTS^{\bullet+}$ and DPPH assays (Table 3.2). Corn-fed yolks showed significantly higher ABTS and DPPH values than wheat-fed yolk samples. All cooked samples had lower RSA values than the raw sample with the exception of the fried corn-fed yolk. The decreasing trend was different from the TPCs, indicating that there are some other compounds contributing to the antioxidant activity. In ABTS and DPPH assays for the corn-fed egg yolks, microwaving appears to be the most deleterious method to the antioxidant activity compared to boiling and frying. This could be ascribed to the degradation of antioxidative compounds due to high and uniform heat generation during microwave cooking. A study conducted by Faller & Fialho (2009) on the DPPH RSA of several vegetables (carrot, onion, potato, broccoli, white cabbage) also showed that both boiling and microwaving reduced the RSA. Zhang & Hamauzu (2004) also reported that microwave and conventional cooking reduced the antioxidant activity of broccoli; they stated that the decrease might be due partially to leaching or attributable to loss of the primary structure of the antioxidative compounds.

3.3.3. Phenolic Acids in Egg Yolk

Egg yolk extracts were analyzed by HPLC-DAD-MS/MS to characterize the phenolic compounds. HPLC-DAD chromatograms obtained for yolk samples did not show the presence of any phenolic acids. Ferulic and isoferulic acids were detected in both types of yolk extracts in

multiple reaction monitoring (MRM) mode. Since these compounds were found only in trace quantities, no attempts were made for quantification. It appears that, under natural conditions, deposition of simple phenolic acids into the chicken egg yolk is very limited and this is in agreement with previous observations (Johnson *et al*., 2008). This could be due to the more hydrophilic nature of the simple phenolic acids compared to other polyphenols such as, isoflavones, lignans, coumestrol etc., which have been reported to occur in chicken eggs (Kuhnle *et al*., 2008).

Interestingly, while phenolic acids could be detected only in trace amounts, two unknown compounds of aromatic nature were found in considerable quantities in all yolk samples and in poultry feed samples by HPLC-DAD. LC/MS-MS analyses revealed that these are tryptophan and tyrosine (Figure 3.1), two aromatic amino acids with reported antioxidant activities (Xu *et al*., 2009; Yan *et al*., 1999). Therefore, it was assumed that these compounds contribute to the antioxidant capacity of egg yolk. To confirm this, tryptophan and tyrosine were isolated using preparative HPLC and subjected to antioxidant assays.

3.3.4. Antioxidant Activity of Tryptophan (Trp) and Tyrosine (Tyr) from Egg Yolk

Yolk Tyr and Trp showed high $ABTS^{\bullet+}$ and ORAC values (Table 3.3). The activities of yolk samples were compared with that of Trp and Tyr standards. ORAC values of Trp and Tyr from yolk were 25.74 ± 1.76 µmol TE/mg and 24.20 ± 3.94 µmol TE/mg, respectively and these values are comparable to values reported earlier (Huang, Majumder, & Wu, 2010). $ABTS^{\bullet+}$ values of yolk Tyr and Trp were 20.58 ± 1.27 µmol TE/mg and 19.23 ± 1.43 µmol TE/mg, respectively, indicating the high radical scavenging ability. Tyr is an aromatic amino acid (see Figure 3.1) and structurally related to *p*-coumaric acid, a phenolic acid with high antioxidant

activity, except that Tyr contains an amino group. The aromatic nature of its structure is considered to be the major factor contributing to its antioxidant activity. It was proposed that the antioxidant activity of Trp is mainly due to its indole group (Galisteo & Herraiz, 2004). Trp has been studied as an antioxidant in different food materials such as yacon (Yan *et al*., 1999) and potato (Xu *et al*., 2009). A recent study showed that Trp in mother's milk has very high antioxidant activity, particularly a high free radical scavenging ability and it is used to increase the antioxidant propertie

s of infant formula (Tsopmo *et al*., 2009). Apart from being an essential amino acid and a precursor of neuromediators such as serotonin, Trp may exert some important biological functions through its high antioxidant activity. To the best of our knowledge, this is the first study to report the antioxidant activities of free tryptophan and tyrosine in egg yolk.

Table 3.3 ORAC and ABTS values of tryptophan and tyrosine [n]

Sample	ORAC value (μmol TE/mg)	ABTS value (μmol TE/mg)
Tryptophan	32.14 ± 0.56	16.02 ± 1.29
Tryptophan from egg yolk	25.74 ± 1.76	19.23 ± 1.43
Tyrosine	24.91 ± 0.24	11.49 ± 0.87
Tyrosine from egg yolk	24.20 ± 3.94	20.58 ± 1.27

[n] All data were expressed as mean ± standard deviation

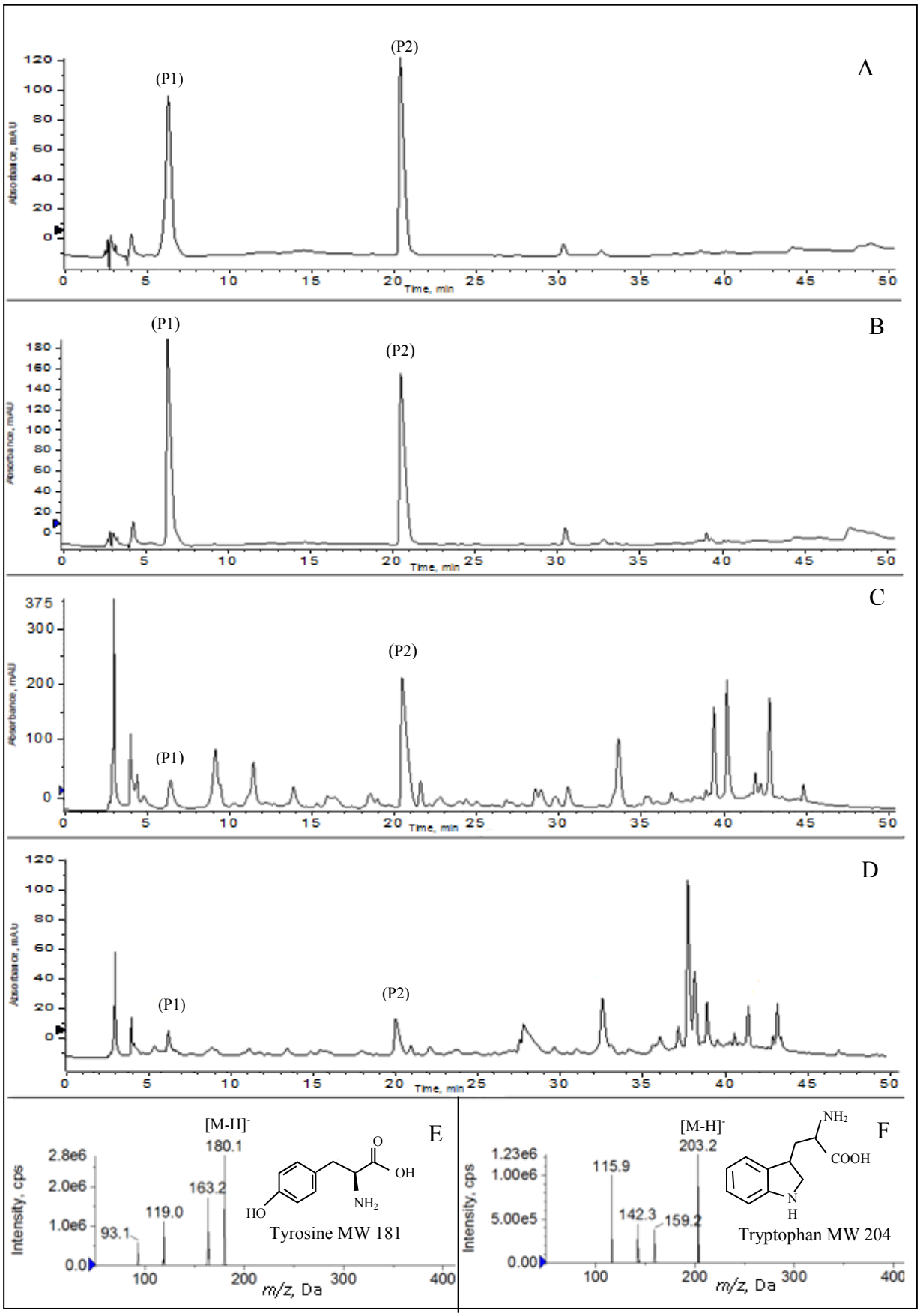
A
(P1)
(P2)
Absorbance, mAU
Time, min
B
(P1)
(P2)
Absorbance, mAU
Time, min
C
(P1)
(P2)
Absorbance, mAU
Time, min
D
(P1)
(P2)
Absorbance, mAU
Time, min
E
[M-H]-
180.1
163.2
119.0
93.1
Intensity, cps
m/z, Da
Tyrosine MW 181
F
[M-H]-
203.2
115.9
142.3
159.2
Intensity, cps
m/z, Da
Tryptophan MW 204

Figure 3.1. Wheat fed egg yolk extract (A), Corn fed egg yolk extract (B), wheat based poultry feed extract (C) and corn based poultry feed extract (D) showing the common peaks identified as tyrosine (P1) and tryptophan (P2). Product ion spectra of tyrosine *m/z* 181 (E) and tryptophan *m/z* 204 (F). HPLC-MS/MS conditions are as described in the text.

3.3.5. Free Amino Acids in Egg Yolk

The composition of free amino acids in egg yolk was analyzed after in-column derivatization with *o*-phthaldialdehyde (OPA). The respective chromatograms are shown in Figure 3.2. The contents of free amino acids in raw and cooked samples in both wheat-fed and corn-fed yolks are shown in Table 3.4.

The contents of total free amino acids were 10081.0 and 10009.5 μg/g of dry yolk in wheat-based and corn-based yolks, respectively. No difference in the composition of amino acids between corn-fed and wheat-fed eggs was observed. Essential amino acids account for ~ 46-47% while aromatic amino acids account for ~11% of the total amino acids; arginine was the most abundant amino acid (~1500 μg/g of dry yolk) in all samples followed by glutamic acid (~1200 μg/g of dry yolk) and lysine (~900 μg/g of dry yolk). Although the presence of free amino acids in eggs was observed as early as in 1927, there is scarce information available in the literature on the quantity of individual free amino acids in egg yolk (Bezelgues, Morgan, Palomo, Crosset-Perrotin, & Ducret, 2009; Ducay, Kline, & Mandeles, 1960). The total free amino acid in egg yolk was reported to be ~ 40 μM/mL of fresh yolk (Ducay *et al*., 1960; Fitzsimmons & Waibel, 1968).

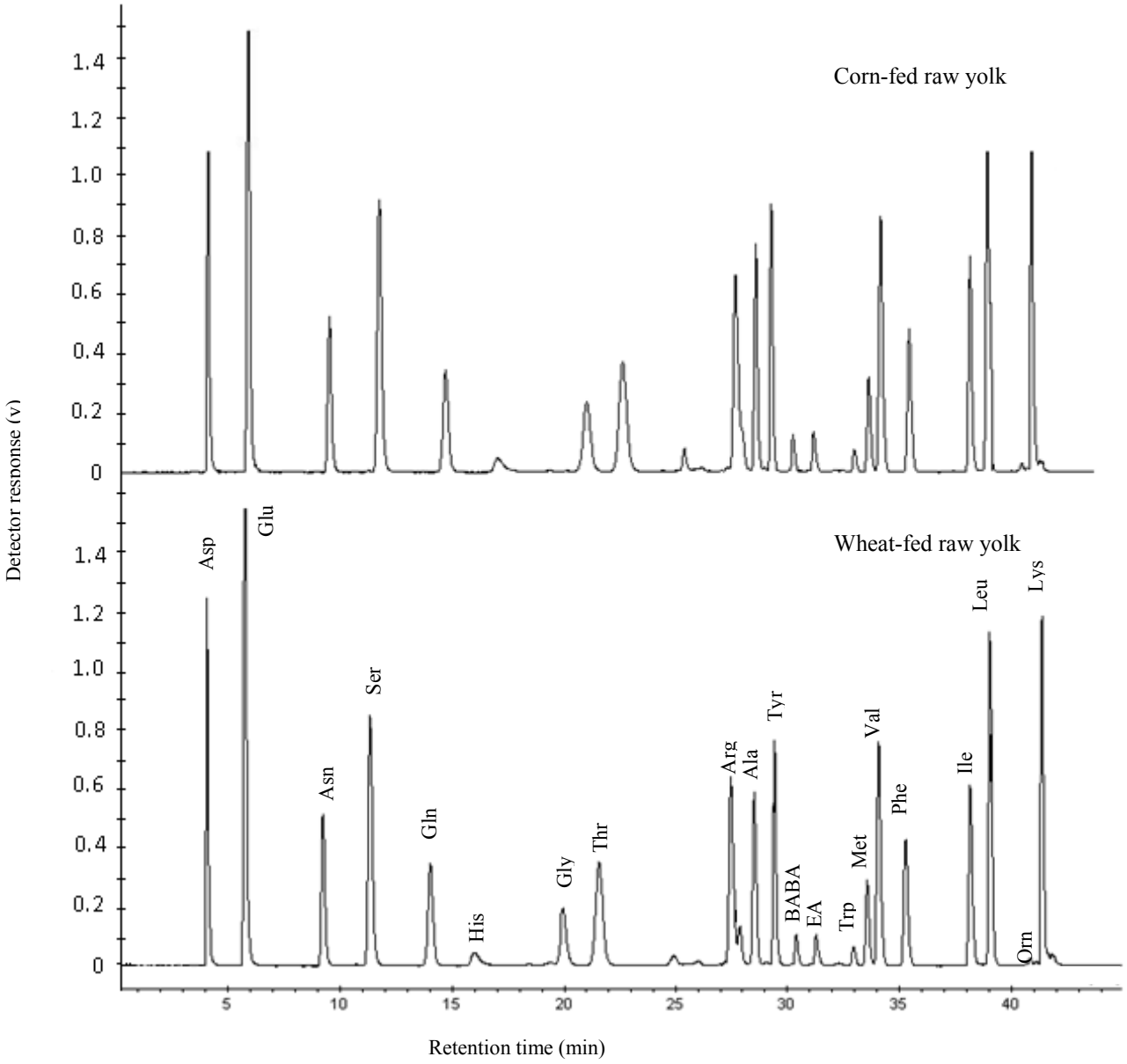

Figure 3.2. HPLC chromatograms showing the free amino acid profiles in wheat-fed and corn-fed raw egg yolk

Table 3.4 Free amino acid contents of the raw and cooked yolk samples (µg/g of dry weight) [n]

Amino Acids	Corn fed eggs				Wheat fed eggs			
	Raw	Boiled	Fried	Microwaved	Raw	Boiled	Fried	Microwaved
Asp	499.5 ± 4.6 ^{ab}	489.1 ± 24.9 ^{ab}	492.2 ± 25.2 ^{ab}	448.2 ± 5.2 ^{cd}	520.4 ± 6.5 ^{a}	447.1 ± 3.8 ^{cd}	476.1 ± 3.3 ^{bc}	435.4 ± 4.5 ^{d}
Glu	1171.9± 10.7^{bc}	1214.8 ± 54.9 ^{b}	1158.4 ± 1.8 ^{c}	1153.5 ± 4.8 ^{c}	1280.2 ± 11.6 ^{a}	1153.0 ± 2.1 ^{c}	1175.5 ± 0.6 ^{bc}	1151.6 ± 15.5 ^{c}
Asn	290.6 ± 2.5 ^{ab}	286.9 ± 15.5 ^{bc}	280.3 ± 13.9 ^{bcd}	266.3 ± 3.7 ^{d}	306.5 ± 3.2 ^{a}	266.8 ± 2.7 ^{d}	282.9 ± 4.3 ^{bcd}	267.7 ± 0.4 ^{cd}
Ser	603.4 ± 6.4 ^{ab}	595.1 ± 37.2 ^{ab}	583.9 ± 19.6 ^{abc}	562.8 ± 7.2 ^{bcd}	610.4 ± 9.5 ^{a}	534.6 ± 2.8 ^{d}	574.4 ± 12.8 ^{abcd}	551.6 ± 3.8 ^{cd}
Gln	345.6 ± 3.7 ^{b}	285.6 ± 15.0 ^{e}	318.1 ± 12.6 ^{cd}	299.4 ± 4.4 ^{de}	385.3 ± 8.2 ^{a}	305.3 ± 2.2 ^{d}	335.7 ± 1.6 ^{bc}	336.6 ± 2.8 ^{bc}
His	145.4 ± 0.1 ^{a}	137.0 ± 8.0 ^{b}	128.5 ± 4.3 ^{bc}	123.0 ± 2.2 ^{c}	149.8 ± 3.3 ^{a}	121.3 ± 0.1 ^{c}	134.0 ± 1.2 ^{b}	122.5 ± 0.5 ^{c}
Gly	251.8 ± 10.0 ^{a}	225.6 ± 12.6 ^{bc}	236.0 ± 18.7 ^{ab}	213.7 ± 6.9 ^{c}	206.3 ± 1.7 ^{cd}	179.0 ± 1.3 ^{e}	186.4 ± 3.3 ^{de}	180.0 ± 0.5 ^{e}
Thr	573.4 ± 4.9 ^{a}	542.9 ± 27.8 ^{bc}	531.1 ± 18.5 ^{cd}	502.3 ± 6.7 ^{d}	564.2 ± 6.0 ^{ab}	503.0 ± 2.0 ^{d}	515.9 ± 5.4 ^{cd}	503.2 ± 1.7 ^{d}
Arg	1584.3 ± 9.1 ^{a}	1602.4 ± 97.9 ^{a}	1494.3 ± 36.4 ^{b}	1439.7± 12.2^{bc}	1390.7 ± 1.4 ^{c}	1228.6 ± 7.7 ^{de}	1271.6 ± 13.1 ^{d}	1172.8 ± 7.2 ^{e}
Ala	625.5 ± 5.6 ^{a}	616.0 ± 32.1 ^{ab}	603.3 ± 23.2 ^{abc}	584.4 ± 8.0 ^{bc}	615.7 ± 1.2 ^{ab}	541.5 ± 2.0 ^{d}	575.9 ± 9.1 ^{cd}	547.7 ± 2.6 ^{d}
Tyr	688.6 ± 2.0 ^{a}	656.5 ± 33.3 ^{a}	613.1 ± 22.9 ^{b}	560.7 ± 5.8 ^{c}	690.6 ± 3.0 ^{a}	600.0 ± 5.7 ^{b}	599.6 ± 10.3 ^{b}	540.1 ± 3.2 ^{c}
Trp	44.1 ± 0.2 ^{a}	40.1 ± 2.5 ^{b}	36.0 ± 2.3 ^{c}	32.7 ± 0.4 ^{d}	44.5 ± 0.1 ^{a}	36.1 ± 0.2 ^{c}	35.6 ± 0.9 ^{c}	31.1 ± 0.5 ^{d}
Met	160.3 ± 1.2 ^{ab}	152.2 ± 8.7 ^{bc}	145.0 ± 5.3 ^{cde}	138.88 ± 1.8 ^{de}	166.5 ± 1.7 ^{a}	140.9 ± 0.1 ^{de}	147.9 ± 2.2 ^{cd}	135.7 ± 0.2 ^{e}
Val	659.2 ± 3.8 ^{ab}	632.0 ± 34.5 ^{bc}	609.2 ± 25.9 ^{cd}	580.83 ± 5.3 ^{d}	679.4 ± 6.5 ^{a}	590.6 ± 1.7 ^{d}	616.6 ± 6.7 ^{cd}	589.2 ± 1.5 ^{d}
Phe	338.6 ± 0.7 ^{a}	316.8 ± 16.5 ^{b}	297.3 ± 11.4 ^{c}	271.15 ± 2.9 ^{de}	352.3 ± 0.2 ^{a}	286.4 ± 1.4 ^{cd}	292.9 ± 4.6 ^{c}	263.4 ± 0.4 ^{e}
Ile	388.3 ± 0.2 ^{b}	369.8 ± 19.1 ^{bc}	358.7 ± 15.9 ^{cd}	332.45 ± 4.7 ^{e}	418.5 ± 5.2 ^{a}	345.6 ± 2.7 ^{de}	365.7 ± 5.8 ^{bcd}	334.2 ± 0.8 ^{e}
Leu	744.0 ± 3.2 ^{ab}	706.4 ± 35.0 ^{c}	550.0 ± 16.9 ^{bc}	629.40 ± 9.8 ^{e}	769.7 ± 6.7 ^{a}	654.2 ± 3.2 ^{de}	669.5 ± 9.7 ^{d}	619.3 ± 0.6 ^{e}
Orn	19.87 ± 0.4 ^{b}	17.50 ± 1.6 ^{bc}	16.89 ± 0.4 ^{cd}	14.03 ± 0.6 ^{d}	23.49 ± 2.8 ^{a}	16.51 ± 0.1 ^{cd}	20.16 ± 0.5 ^{b}	16.70 ± 0.4 ^{cd}
Lys	874.2 ± 1.7 ^{ab}	876.5 ± 39.9 ^{ab}	832.2 ± 28.6 ^{bc}	814.4 ± 13.0 ^{cd}	905.7 ± 2.1 ^{a}	785.7 ± 7.9 ^{cd}	829.0 ± 19.5 ^{bc}	774.3 ± 2.1 ^{d}
Total	10009.5 ± 70.6^{a}	9764.2 ± 515.4^{ab}	9285.2 ± 303.4^{bc}	8968.4 ± 105^{cd}	10081.0 ± 8.1^{a}	8737.1 ± 49.6^{d}	9106.1 ± 114.4^{cd}	8573.9 ± 48.8^{d}

[n] Results are means ± Standard deviation (n=6), [a-e] values with different superscripts within a row indicate significant differences at $P < 0.05$ separated by Duncan's multiple range test.

The total amino acid content of yolk samples is significantly reduced during all types of cooking treatments except for the boiled egg yolk of corn-fed eggs. There is a trend that microwave caused greater reduction of the total amino acid content than other methods. A significant decrease in amino acids during steaming of faba beans (Ziena, Youssef, & El-Mahdy, 1991) and "ginseng" herb (Cho *et al*., 2008) was attributed to Maillard reactions. Most of the amino acids, including aromatic amino acids, were significantly reduced in quantity during cooking; microwave cooking caused the highest losses in both type of samples (Table 3.4). A previous study on conventional and microwave cooking of pork, beef and lamb showed that most essential amino acids including leucine, isoleucine, phenylalanine and methionine were affected during cooking (Baldwin, Korschgen, Russel, & Mabesa, 1976).

3.4. Conclusions

In the present study, egg yolk was initially screened to determine phenolic compounds by reversed-phase HPLC-DAD-MS/MS. Transfer of simple phenolic acids to egg yolk appeared to be minimal under natural conditions and only ferulic acid was detected in trace amounts in the MRM scan mode. Two unknown compounds of an aromatic nature found in considerable amounts in all yolk samples were identified as tryptophan and tyrosine. These compounds are the major contributors to the total antioxidant capacity of egg yolk extracts. The total phenolic content and total antioxidant capacity were affected significantly during cooking; microwave cooking caused the highest reduction in antioxidant capacity while frying reduced the total phenolic content to the highest extent. For the first time, our results showed that free amino acids contribute to the total antioxidant capacity of egg yolk and domestic cooking methods significantly reduced the quantity of free amino acids in egg yolk, with microwaving caused the

highest losses. The onset and progression of many forms of chronic diseases including cardiovascular diseases are highly related to oxidative stress. The results of our studies suggest that eggs contain significant quantities of antioxidants in addition to its well-known nutrients. Consumption of cholesterol-rich foods such as eggs was once indicated as a possible causing factor for cardiovascular diseases; findings from the present study might even give rise to a paradigm change in the nutritional evaluation of eggs.

3.5. References

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CHAPTER 4 - Effect of Domestic Cooking Methods on Egg Yolk Xanthophylls[2]

[2]A version of this chapter has been published: Nimalaratne, C., Lopes-Lutz, D., Schieber, A., & Wu, J. (2012). Effect of Domestic Cooking on Egg Yolk Carotenoids. *Journal of Agricultural and Food Chemistry*. 60(51):12547-12552.

CHAPTER 4

4.1. Introduction

Xanthophylls, the yellow colour pigments of egg yolk, have long been associated with the quality of eggs by consumers (Gordon, Bauernfeind, & Furia, 1982). Later, with more detailed understanding of the link between xanthophylls and human health, they became an important class of bioactive compounds (Stahl & Sies, 2005; Voutilainen, Nurmi, Mursu, & Rissanen, 2006). Among many food types, egg yolk is considered as one of the important sources of xanthophylls with higher bioavailability than other common sources such as dark-green leafy vegetables (McNamara, 2006; Schlatterer & Breithaupt, 2006). The xanthophyll carotenoids, especially lutein and zeaxanthin, are known to be accumulated in the macular region of the human retina and play an important role in human health especially in preventing age-related macular degeneration, the main reason for vision loss of the elderly in western countries (Handelman, Nightingale, Lichtenstein, Schaefer, & Blumberg, 1999; McNamara, 2006; Ribaya-Mercado & Blumberg, 2004). Several studies reported the elevation of plasma lutein and zeaxanthin levels followed by diets containing egg yolks (Handelman *et al*., 1999; Surai, MacPherson, Speake, & Sparks, 2000).

An average egg yolk usually contains about 175-400 µg of lutein and about 200-300 µg zeaxanthin, but this could be significantly affected by hens' feed composition (Handelman *et al*., 1999). In commercial poultry farming, synthetic xanthophylls are permitted to be used in poultry feed in accordance with the maximum allowable limits. In organic farming, as synthetic additives are not permitted, xanthophyll rich plants are used to obtain the desired amounts in the eggs (Breithaupt, 2008). In designer eggs, which are enriched with desired compounds through modified poultry feed, lutein content was reported as high as 1.91 mg per yolk (Surai *et al*., 2000).

CHAPTER 4

Xanthophylls are dihydroxy carotenoids with long conjugated polyene chain which predominantly exist in nature as all-*(E)* isomeric form. They are susceptible to processing conditions such as heat, light and oxygen and can be converted into their *cis* isomeric forms, which may result in changes of their functionality and bioavailability (Dugave & Demange, 2003; Schieber & Carle, 2005). Effect of thermal processing on carotenoids in various fruits and vegetables such as orange, broccoli, corn, spinach etc. has been studied in the past years (Aman, Schieber, & Carle, 2005; Aman *et al*., 2005; Gama & de Sylos, 2007; Updike & Schwartz, 2003). However, very few studies are reported the effect of cooking on egg yolk xanthophyll composition. One study showed that boiling of eggs decreased the xanthophyll content of the yolk with 20% loss of lutein and, the content of other xanthophylls reduced from 10% to 20% (Schlatterer & Breithaupt, 2006). Pasteurization of liquid egg yolk has no influence on the xanthophyll content while significant reductions were observed during the storage time regardless of the storage temperature being -18 °C or 20 °C (Wenzel, Seuss-Baum, & Schlich, 2010; Wenzel, Seuss-Baum, & Schlich, 2011). Another study found that boiling of designer eggs did not change the lutein concentration in egg yolk (Surai *et al*., 2000). There is limited information found in the literature dealing with the effects of different home cooking methods such as boiling, microwaving, frying etc. on egg yolk xanthophylls. Therefore, it was of interest to study how different cooking methods may affect the content and composition of egg yolk xanthophylls.

4.2. Materials and methods

4.2.1. Materials and Chemicals

Light petroleum ether, methanol, acetone, ethyl acetate and TBME (*tert*-butyl methyl ether) were purchased from Fisher Scientific (Ottawa, ON, Canada) and were of HPLC grade. Lutein (xanthophyll from marigold), zeaxanthin, canthaxanthin and β-apo-8'-carotenal, silica gel 60 (0.063-0.200 mm) and butylated hydroxyl toluene (BHT) were purchased from Sigma (Oakville, ON, Canada). β-apo-8'-carotenoic acid ethyl ester was obtained from CaroteNature (Lupsingen, Switzerland). (*Z*)-isomers were obtained by iodine-catalyzed photoisomerization of the (all-*E*)-carotenoids (Aman *et al*., 2005).

4.2.2. Iodine-catalyzed photoisomerization to obtain *(z)*-isomers

Stock solutions of (all-*E*)-compounds were prepared to a concentration of 500 ug/mL in TBME:Methanol (3:1 v/v) containing 0.1% BHT and stored in amber color glassware. To obtain (*Z*)-isomers of reference compounds, the following procedure, adapted from Aman *et al*., (2005) was used with minor modifications. For 200 uL of (all-*E*) standards, 150 uL iodine in hexane (c = 40 umol/L) (to obtain a final iodine concentration about 1–2% (w/w) of the carotenoid) was added. The solutions were exposed to fluorescent light (four fluorescent lamps: L36W/76 nature de luxe, Osram, Munich, Germany) for 30 min at room temperature. The illumination intensity was measured using a luxmeter (3900 lx) (Mavolux-digital, Gossen, Nuernberg, Germany). The solutions were then washed twice with $Na_2S_2O_3$ solution (1 mol/L) to remove excess iodine and evaporated under nitrogen gas. The residues were dissolved in a mixture of TBME:methanol (3:1, v/v) containing 0.1% BHT and made up to 1 mL (final concentration 100 ug/mL).

4.2.3. Preparation of samples

Eggs (n = 48) were purchased from a local supermarket (Edmonton, AB, Canada) and all experiments were performed at least two weeks prior to the expiry date. Eggs were divided into four equal sets and three sets were subjected to different cooking treatments, boiling, microwaving and frying, and the fourth was used as the control.

4.2.4. Cooking of eggs

One set of whole shell eggs were placed in a saucepan as a single layer, with water up to 1-2 inches above the eggs, and boiled for 10 min. After boiling, they were placed under running tap water for 5 min, peeled and yolks were separated from whites. For microwaving, whole egg without the shell was placed in a microwavable glass bowl and cooked in a household microwave oven (model DMW 113w, Danby Products, Ontario, Canada) for 90 seconds at cooking level (1100 W, 2450 MHz); egg yolks were carefully separated from whites. Another set of eggs were fried using a frying pan (model SK200TY non-stick frying pan, Black & Decker Canada Inc., Brockville, ON, Canada) preheated to 205 °C. Whole eggs were fried for 6 min (3 min each side) and the yolks were separated from whites. Raw egg yolks were used as a control. To prepare raw yolk samples, egg yolks were manually separated from whites and wiped with a filter paper to remove adhered albumins. Cooked and raw egg yolks were pooled, homogenized and placed immediately at -20 °C and then subjected to freeze drying in containers impermeable to light. Freeze-dried samples were ground to obtain a fine powder. The processed samples were stored in the dark at -20 °C in airtight sealed plastic containers until analysis.

4.2.5. Extraction of xanthophylls from egg yolks

Yolk samples were extracted according to Schlatterer & Breithaupt (2006) with minor modifications. Approximately 5 g of freeze dried egg yolk powder were weighed into a glass extraction tube covered with aluminum foil to protect xanthophylls from light. The samples were extracted three times (15 mL each) using a ternary solvent mixture (methanol: ethyl acetate: petroleum ether, 1:1:1, v/v/v) with 0.1% BHT, the supernatants were combined and evaporated under nitrogen gas. The oily residue was completely transferred to a 10 mL volumetric flask and made up to the volume with TBME:methanol (3:1 v/v) containing 0.1% BHT. These samples were filtered through 0.45 μm nylon membrane filter and analyzed by HPLC. Dim light conditions and amber color glassware were used throughout the extraction and analysis to protect the xanthophylls from light induced isomerization. Samples were prepared in triplicate.

4.2.6. Chromatography

HPLC-DAD analysis. The samples were analyzed with a Waters 600 HPLC system (Waters, Millford, MA, USA) equipped with a 2702 thermoautosampler, a binary gradient pump and a 2998 photodiode array detector. The reversed-phase separation was performed on a C_{30} reversed-phase column (YMC 250 mm × 4.6 mm, i.d., 5 μm) protected with a C_{18} guard column (20 mm × 3 mm, i.d., 5 μm) operating at 20°C and at a flow rate of 1 mL/min. The compounds were separated with gradient elution as described by Aman *et al*., (2005). Samples were eluted with methanol/TBME/water (92:4:4 v/v/v with 0.1% BHT) as solvent A and methanol/TBME/water (6:90:4 v/v/v with 0.1% BHT) as solvent B using a linear gradient from 100% A to 6% B within 90 min. The injection volume was 20 uL. Monitoring was performed at 450 nm and the analyses were performed in duplicates.

4.2.7. Purification of yolk samples for LC-MS/MS analysis

Yolk extracts were evaporated under nitrogen and reconstituted in 10 mL of acetone and kept at -20 °C overnight. Then, the samples were vacuum filtered using a sintered glass funnel in a freezer compartment at -20°C to remove the crystallized lipids (Rodriguez-Amaya, 2003). The filtrate was evaporated under nitrogen and made up to a volume of 10 mL with TBME/methanol (3:1 v/v with 0.1% BHT). All samples were filtered through 0.45 μm nylon filter before injecting for LC-MS/MS analysis.

4.2.8. LC-(APCI)-MS/MS conditions

For separation and identification of xanthophylls, an Agilent 1200 HPLC system including degasser, binary pump, autosampler, thermostatted column compartment and diode array detector (DAD) (Agilent Technologies, Palo Alto, CA) was connected to a 4000 QTrap mass spectrometer (AB Sciex, Concord, ON, Canada) fitted with an atmospheric pressure chemical ionization (APCI) source. The separation was performed on a YMC C_{30} reversed phase column (Waters XBridge 250 mm × 4.6 mm i.d., 5 μm) and a C_{18} guard column (20 mm × 3 mm, i.d., 5 μm) at a flow rate of 0.5 mL/min. The solvent system and the gradient program were the same as described above. Mass spectrometric data was acquired and processed using Analyst software (version 1.5). The mass spectrometer was operated in positive ionization mode and data were obtained using enhanced MS (EMS) and enhanced product ion (EPI) scans. Mass spectra were recorded over the range from *m/z* of 300 to 600 Da at a scan rate of 1000 Da/s for EMS and 4000 Da/s for EPI scans. The instrument parameters for curtain gas, nebulizer gas (GS1), needle current and ion source temperature were 10 psi, 25 psi, 5 μA and 400 °C, respectively. The optimized values for other MS parameters were as follows: declustering potential (DP) at 36 V,

entrance potential (EP) at 10 V, collision energy (CE) at 21 eV, and collision cell exit potential (CXP) at 12 V.

4.2.9. Quantification

Seven point standard calibration curves were prepared for quantification purposes. Calibration graphs were recorded with sample concentrations ranging from 0.05 to 30.00 ug/mL by plotting the respective analyte peak area against concentration. Both *E*- and *Z*- isomer concentrations were calculated using the corresponding (all-*E*)-standard calibration curves. The limit of detection (LOD) and limit of quantitation (LOQ) were determined by injecting a series of dilute solutions with known concentrations. LOD and LOQ were defined as the signal-to-noise ratio equal to 3 and 10, respectively, according to the International Conference on Harmonization (ICH) Guideline (ICH guidelines, 1997).

4.2.10. Statistical analysis

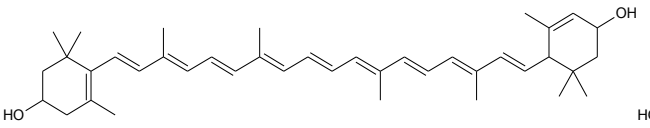
All data were processed by analysis of variance (ANOVA) followed by Tukey's multiple range test using Statistical Analysis System Software (SAS version 9.0, SAS Institute, Cary, NC, USA). Significance of differences was defined at the 5% level ($p < 0.05$).

4.3. Results and discussion

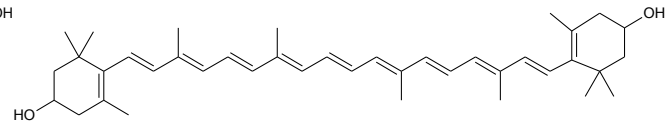
4.3.1. Purification of egg yolk extracts

Egg yolk extracts have very high lipid content and have to be purified before mass spectrometric analysis. Lipids of extracted yolk samples can be removed by fractionating on a silica gel column (Schlatterer & Breithaupt, 2006) or by subjected to filtration in a freezer compartment

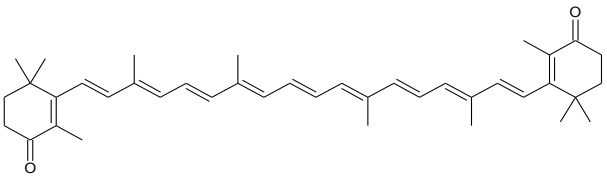
(Rodriguez-Amaya, 2003). Open column chromatography with silica gel column is time consuming and requires a high amount of organic solvents. Therefore, in this experiment, the samples were subjected to filtration in a freezer compartment and this has been proven to be able to remove about 90% of total lipids present in the sample.



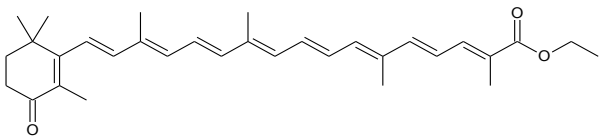
All-*E*-lutein



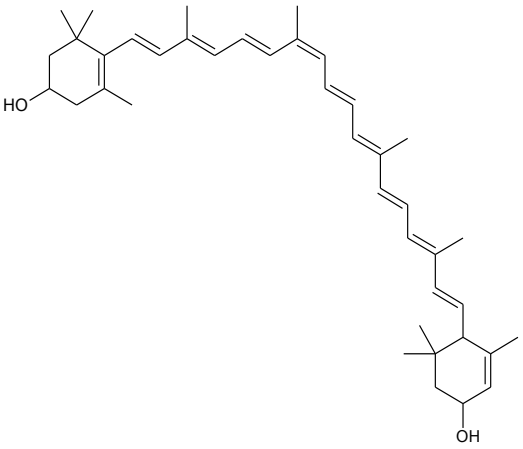
All-*E*-zeaxanthin



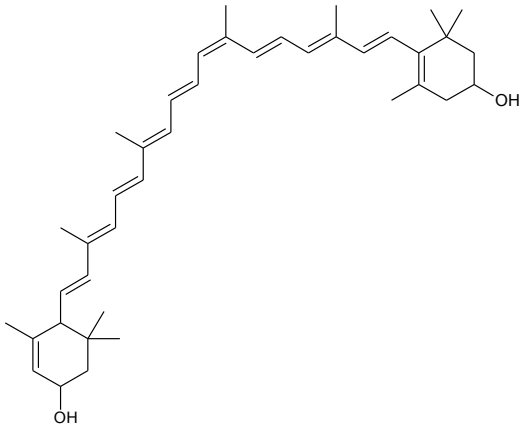
All-*E*-canthaxanthin



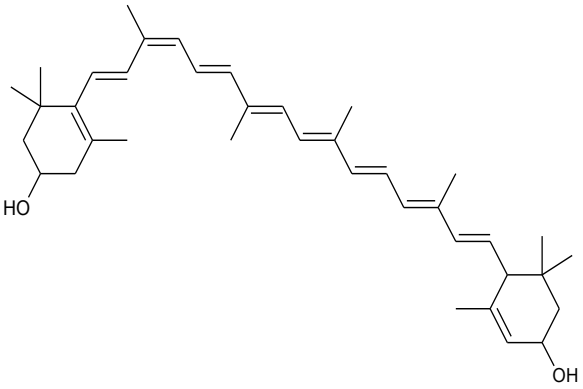
All-*E*- β-apo-8'-carotenoic acid ethyl ester



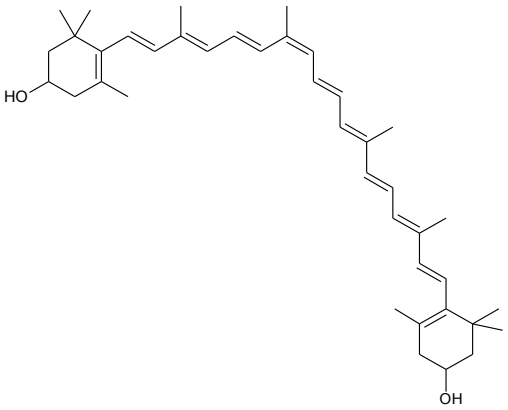
13-*Z*-lutein



13'-*Z*-lutein



9-*Z*-lutein



13-*Z*-zeaxanthin

Figure 4.1. Chemical structure of xanthophylls found in egg yolk

4.3.2. Identification of xanthophylls in egg yolk

A C_{30} reversed-phase column was used to obtain the baseline separation of nine xanthophylls including the *Z*-isomers of lutein, zeaxanthin and canthaxanthin that were found in egg yolk (Figures 4.1 and 4.2). Identification of xanthophylls was based on their UV spectra, retention times, mass spectra, fragmentation pattern and the elution order of the compounds as shown in Table 4.1.

Table 4.1: UV-Visible and mass spectrometric data of xanthophylls in egg yolk obtained by HPLC-DAD-APCI-MS

Peak	Name	λ_{max} (nm)	$[M+H]^+$ (*m/z*)	Fragment ions (*m/z*)
1	13-*Z*-lutein	330, 415, 436, 463	569	477 [M + H – 92], 551 [M + H - 18]
2	13*'*-*Z*-lutein	330, 415, 437, 465	569	477 [M + H – 92], 551 [M + H - 18]
3	13-*Z*-zeaxanthin	337, 422, 442, 465	569	477 [M + H – 92], 551 [M + H - 18]
4	All-*E*-lutein	332, 421, 444, 472	569	477 [M + H – 92], 551 [M + H - 18], 459 [M + H-18-92]
5	*Z*-isomer of canthaxanthin	364, 465	565	547 [M + H - 18], 473 [M + H – 92], 363 [M + H-92-92-18]
6	All-*E*-zeaxanthin	348, 426, 450, 478	569	477 [M + H – 92], 551 [M + H - 18]
7	9-*Z*-lutein	327, 414, 439, 466	569	477 [M + H – 92], 551 [M + H - 18]
8	All-*E*-canthaxanthin	477	565	547 [M + H - 18], 473 [M + H – 92], 363 [M + H – 92-92-18]
9	9'-*Z*-lutein	416, 440, 468	569	477 [M + H – 92], 551 [M + H - 18]
10	All-*E*-β-apo-8'-carotenoic acid ethyl ester	445	461	443 [M + H - 18], 369 [M + H - 92]

Because of their similar mass spectra, identification of *Z*-isomers of lutein and zeaxanthin was mainly based on the UV spectra, retention times and the elution order (Aman *et al*., 2005). All compounds showed precursor ion $[M+H]^+$ as the base peak. Most abundant product ions were $[M+H-18-92]^+$ (loss of water and toluene) and $[M+H-18]^+$ (loss of water). UV/Vis spectra of all-*E* xanthophylls were similar in shape to those of *Z* isomers, but small hypsochromic shifts were observed. As reported previously, introduction of *cis* double bond shifts the maximum absorption to a shorter wavelength (Dugo *et al*., 2006). Compared to all-*E* lutein, 9-*Z*, 9*'*-*Z,* 13-*Z* and, 13*'*-*Z* isomers showed hypsochromic shifts between 4 and 8 nm (Table 4.1). *Cis*-canthaxanthin showed a hypsochromic shift of 12 nm and an additional "*cis* peak" at 364 nm was also present (Nelis *et al*., 1984).

Table 4.2: Limit of detection (LOD) and limit of quantitation (LOQ) for xanthophyll analysis by HPLC-DAD

Analyte	Calibration range (µg/mL)	LOD (µg/L)	LOQ (µg/L)
Lutein	0.10 – 30.0	9.4	31.4
Zeaxanthin	0.20 – 30.0	9.5	31.6
Canthaxanthin	0.05 – 30.0	16.6	55.5
Ethyl ester	0.05 – 20.0	13.3	44.2

Figure 4.2 illustrates the HPLC separation of compounds for the standard mixture of all-*E* xanthophylls and their *Z* isomers (obtained after iodine-catalized photoisomerization of all-*E* standards).

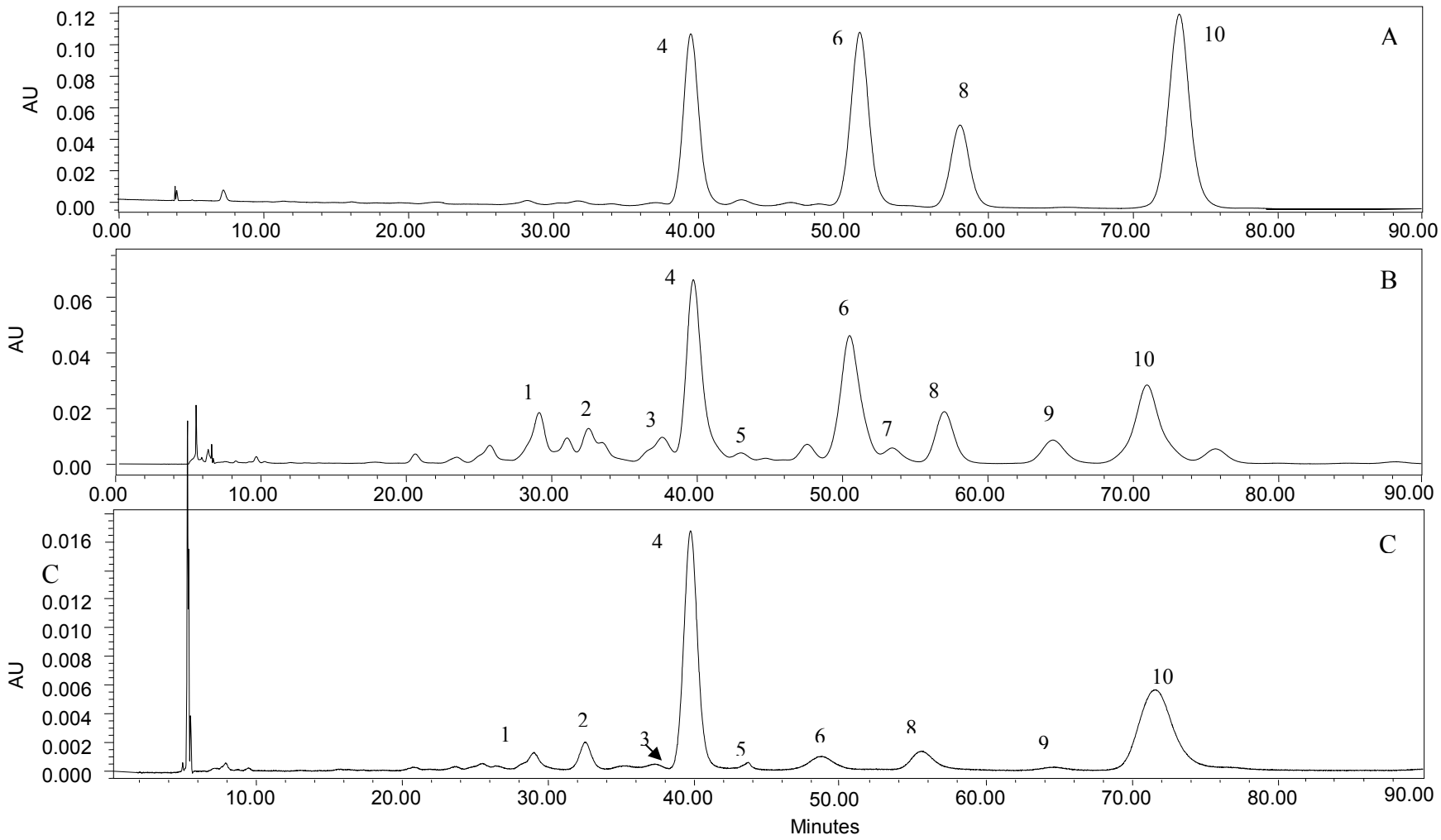


Figure 4.2. HPLC with photodiode-array UV-visible absorbance detection of standard mixture of all-*E* xanthophylls (A), Standard mixture showing the separation of isomers of xanthophylls (B), Extract from egg yolk (raw) showing the separation of xanthophylls (C). Peak identification (1) 13-*Z* lutein, (2) 13'-*Z* lutein, (3) 13-*Z* zeaxanthin, (4) All-*E* lutein, (5) Z isomer canthaxanthin, (6) All-*E* zeaxanthin, (7) 9-*Z* lutein, (8) All-*E* canthaxanthin, (9) 9'-*Z* lutein, (10) All-*E* β-apo-8'-carotenoic acid ethyl ester

Nine xanthophylls were identified in raw egg yolk. Surprisingly, all cooked yolk samples showed xanthophyll profiles similar to that obtained for raw egg yolks, indicating that there was no formation of *cis*-isomers after thermal treatment. The HPLC chromatograms were very similar except the fact that the peaks were of different intensities. This agrees with a previous study on boiled egg yolks (Schlatterer & Breithaupt, 2006), according to which, there were no additional peaks appeared in HPLC chromatograms of boiled yolk extracts compared to that of raw yolk.

4.3.3. Quantification of xanthophylls in yolk samples

The quantitation was based on the standard calibration obtained from UV-detection. Sensitivity of the method was assessed through analyzing the limits of detection and quantitation (LOD and LOQ) for each analyte. The values obtained and the calibration ranges are presented in Table 4.2. The correlation coefficients (R^2) of calibration graphs were >0.999 for all compounds. Sample extracts obtained from egg yolks subjected to different types of domestic cooking, i.e. boiling, frying and microwave cooking were analyzed and the quantities were determined for all nine xanthophylls identified. The extracts of raw and cooked egg yolks were compared for their total xanthophyll content and degree of isomerization.

4.3.4. Effect of cooking on xanthophylls in egg yolk

All-*E*-lutein was the predominant xanthophyll present in egg yolk followed by zeaxanthin, β-apo-8'-carotenoic acid ethyl ester and canthaxanthin. It should be noted that the amount of canthaxanthin in egg yolk (183.43 ± 17.1 µg/ 100) found in this study is considerably lower than those reported previously (Schlatterer & Breithaupt, 2006; M Wenzel *et al*., 2010). This can be attributed to the different poultry feed. As mentioned earlier, the composition and the content of

xanthophylls in egg yolk depend very much on the hen's feed. Depending on the regulation on animal feed, the permitted levels and types of xanthophylls may vary. In the European Union, eight xanthophylls are allowed to be added to the poultry feeding including, lutein, capsanthin, zeaxanthin, β-apo-8'-carotenal, canthaxanthin, β-apo-8'-carotenoic acid ethyl ester, β-cryptoxanthin, and citranaxanthin (Breithaupt, 2008). Currently, in Canada only three xanthophylls are permitted to be added to poultry feeds: lutein (as marigold oil extracts), β-apo-8'-carotenoic acid ethyl ester (crystalline) and canthaxanthin (crystalline) (Feeds Regulations, 1983).

Cooking of eggs did not generate new xanthophyll isomers. All nine xanthophylls detected in cooked yolk extracts were present in raw yolks, but the amounts varied. Table 4.3 summarizes the amounts of xanthophylls present in yolk extracts, the total amount of individual xanthophylls (both *E*- and *Z*- isomers), and the percentage loss or increase of a compound in cooked yolk extracts relative to the raw yolk extracts. After cooking, all-*E* xanthophyll contents decreased while their *Z*- isomers increased. All-*E*-lutein seems to be affected most among the xanthophylls, showing 22.5%, 16.7% and 19.3% reductions in boiled, microwaved and fried yolk extracts respectively. Cooking resulted in a 8 to 15.2% decrease in all-*E*-zeaxanthin, 11.3 to 12.8% in all-*E*-canthaxanthin and 6.9 to 9% decrease in all-*E*- β-apo-8'-carotenoic acid ethyl ester. Similar results were observed in a study on boiled eggs (Schlatterer & Breithaupt, 2006). Total xanthophyll content decreased in all cooked yolk extracts. These losses might reflect a possible degradation of xanthophylls owing to the time-temperature regime. Degradation causes irreversible loss of the compounds due to oxidation and subsequent formation of volatile compounds. The effects and products of thermal degradation were studied only recently and to a

limited extent (Fratianni, Cinquanta, & Panfili, 2010; Rios, Fernández-García, Mínguez-Mosquera, & Pérez-Gálvez, 2008; Zepka & Mercadante, 2009).

Table 4.3: Quantities of each compound in raw and processed samples

		µg/ 100g of egg yolk						
		Raw egg yolk	**Boiled egg yolk**	**% change**	**Microwaved egg yolk**	**% change**	**Fried egg yolk**	**% change**
	13-*Z*-Lutein	67.47 ± 12.9	74.74 ± 10.4	10.8	71.13 ± 8.5	5.4	74.31 ± 7.8	10.1
	13′-*Z*-Lutein	85.56 ± 2.7	93.47 ± 12.5	9.2	94.11 ± 19.42	10.0	92.51 ± 16.6	8.1
	All-*E*-Lutein	1102.71^{a} ± 159.9	854.70^{b} ± 67.6	-22.5	918.48^{b} ± 69.5	-16.7	890.35^{b} ± 44.7	-19.3
	9'-*Z*-Lutein	26.56 ± 6.6	28.19 ± 3.0	6.1	27.42 ± 2.0	3.2	30.09 ± 2.0	13.3
Total lutein		1282.3 ± 182.1	1051.10 ± 93.5	-18.0	1111.13 ± 99.42	-13.3	1087.26 ± 71.1	-15.2
	13-*Z*-Zeaxanthin	79.12 ± 8.5	89.70 ± 9.9	13.4	86.78 ± 22.1	9.7	89.51 ± 12.2	13.1
	All-*E*-Zeaxanthin	560.70 ^{a} ± 131.4	475.73 ^{b} ± 34.6	-15.2	515.60 ^{ab} ± 58.6	-8.0	511.84 ^{ab} ± 50.1	-8.7
Total zeaxanthin		639.8 ± 139.9	565.43 ± 44.5	-11.6	602.38 ± 80.7	-5.9	601.34 ± 62.3	-6.0
	Z-Canthaxanthin	18.59 ^{a} ± 5.2	28.23 ^{b} ± 5.2	51.8	27.29 ^{b} ± 3.12	46.8	27.84 ^{b} ± 4.4	49.7
	All-*E*-Canthaxanthin	174.84 ± 11.9	152.52 ± 5.0	-12.8	155.11 ± 10.9	-11.3	154.50 ± 14.9	-11.6
Total canthaxanthin		183.43 ± 17.1	180.75 ± 10.2	-6.6	182.4 ± 14.02	-5.7	182.34 ± 19.3	-5.7
	All-*E*- β-apo-8'-carotenoic acid ethyl ester	432.52 ^{a} ± 18.4	393.54 ^{b} ± 17.9	-9.0	402.88 ^{ab} ± 36.6	-6.9	396.97 ^{ab} ± 17.5	-8.2

(n= 6); $^{a-b}$ different letters in same row, denote significant difference (p <0.05)

Statistical analysis revealed that the effects of the three cooking methods, boiling, frying and microwaving on yolk xanthophylls are not significantly different among each other. Yet, all three cooking methods cause significant losses of all-*E*-lutein, while boiling causes considerable losses of all-*E*-zeaxanthin and all-*E*-β-apo-8'-carotenoic acid ethyl ester. Except for decreased all-*E*-lutein and increased *Z*-canthaxanthin contents, microwave cooking and frying cause no significant effect on yolk xanthophylls. With respect to the types of stereoisomers, both 13-*Z* and 9-*Z* isomers increased after thermal processing. 13-*Z* isomers were the predominant form of *Z* isomers of lutein and zeaxanthin found in egg yolk and the amounts increased with heating. It has been observed in several studies that heating causes the formation of 13-*Z* isomers of xanthophylls (Chen, Peng, & Chen, 1995; Marx, Stuparic, Schieber, & Carle, 2003).

In this study, the effect of three different types of domestic cooking on yolk xanthophylls was investigated. Cooking caused reductions in the quantity of all-*E* xanthophylls and increased Z-isomers. Boiling significantly reduced the amounts of all-*E*-lutein, all-*E*-zeaxanthin and all-*E*-canthaxanthin, whereas, microwave cooking and frying did not show significant losses except for all-*E*-lutein. The total xanthophylls losses were ranged approximately from 6 to18%. So far, the effect of thermal processing of egg yolk has not been studied in detail. Therefore, the results present in this study will help improving the current knowledge on the effects of cooking on yolk xanthophylls and thus may be useful in assessing the dietary intake of xanthophylls. Furthermore, the analytical method presented here could be useful in assessing the xanthophyll profiles and contents of egg-containing products. Currently, we are working on developing improved methods with shorter analysis time and higher sensitivity using fast liquid chromatography coupled with tandem mass spectrometry.

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CHAPTER 5 - Stepwise Extraction of Antioxidants from Egg Yolk

CHAPTER 5

5.1. Introduction

Egg yolk is a rich source of bioactive compounds such as proteins, phospholipids, vitamins, minerals, and carotenoids which may act as radical antioxidants, immunomodulators, anti-microbials to provide protective effects against various chronic diseases (Anton, Nau, & Nys, 2006). Antioxidants are increasingly recognized for their potential to reduce the risk of oxidative damage from free radicals and oxidants (Carocho & Ferreira, 2013; Halliwell, 1996). This not only applies to human health, but also in protecting food components from oxidation and quality deterioration as well (Shahidi, 2000). Free radicals, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated as a result of normal cellular metabolism and perform as essential part of bodily defence mechanism (Halliwell, 1994; Valko *et al*., 2007). However, a disturbed balance between these free radicals and enzymatic/non-enzymatic antioxidants in the body in favor of the former can result in oxidative stress and can cause damage to biomolecules such as DNA (Sies, 1986). Use of antioxidants are shown to be effective against oxidative damage in food systems as well as *in vivo* (Carocho & Ferreira, 2013).

Free aromatic amino acids, tryptophan and tyrosine have previously been found as the major contributors to the antioxidant activity of egg yolk (Nimalaratne, Lopes-Lutz, Schieber, & Wu, 2011). In addition, egg yolk contains carotenoids, phosvitin and vitamin E, which can contribute to its total antioxidant activity. Carotenoids and vitamin E in hens' feed can be transferred into the egg yolk and therefore the amounts present in yolk are largely depend on the feed composition (Surai & Sparks, 2001). Lutein and zeaxanthin are the main egg carotenoids and form a major part of macular pigment (Bone, Landrum, & Tarsis, 1985). The singlet oxygen and radical scavenging activity of lutein and zeaxanthin is considered as the major mechanism for their beneficial effects against light-induced oxidative damage in eye macular, in particular,

against the age-related macular degeneration (AMD) (Böhm, Edge, & Truscott, 2012; Li, Ahmed, & Bernstein, 2010). Vitamin E is a well-known for *in vitro* and *in vivo* radical scavenging activities (Niki, 2014) and believed to play a role in prevention of atherosclerosis through inhibition of oxidation of low-density lipoprotein (Stephens *et al*., 1996). Inclusion of vitamin E in hens' diet protect against lipid oxidation in omega-3 enriched eggs (Meluzzi, Sirri, Manfreda, Tallarico, & Franchini, 2000). Phosvitin is a phosphoprotein with an excellent iron binding capacity and therefore, considered as a potential natural antioxidant. The antioxidant activity of phosvitin has been studied (Volk, Ahn, Zeece, & Jung, 2012) and also its applicability as an antioxidant in food industry (Jung *et al*., 2012; Lee, Han, & Decker, 2002).

The presence of antioxidants in egg yolk is established; however a systematic approach for extraction of antioxidants has not been reported. This study was designed to determine the total antioxidant activity of egg yolk resulted from different yolk compounds predominantly from free amino acids (FAAs), carotenoids, phosvitin and vitamin E. A stepwise extraction method was developed to identify the degree of contribution from each antioxidant compound to the total activity. Eggs are usually eat in cooked form and different cooking methods, such as frying, boiling, scrambling eggs may change the quantity and chemical properties of these compounds affecting their antioxidant activity. Thus, this also aims to find the effect of different home cooking methods (boiling, frying and scrambling) on the quantity and antioxidant activity.

5.2. Materials and methods

5.2.1. Chemicals

Light petroleum ether, *n*-hexane, methanol, acetone, ethyl acetate, TBME (*tert*-butyl methyl ether) and ethanol were purchased from Fisher Scientific (Ottawa, ON, Canada). Fluorescein

disodium and trolox were obtained from Acros Organics (Morris Plains, NJ). Lutein (xanthophyll from marigold), zeaxanthin, trichloroacetic acid, 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH), and anhydrous monobasic sodium phosphate, were obtained from Sigma–Aldrich (Oakville, ON, Canada). Vitamin E standard ((±)-α-tocopherol (≥95.0% purity)) was purchased from Calbiochem (Darmstadt, Germany).

5.2.2. Processing of Eggs

Fresh eggs (*n*=40) were obtained from the Poultry Research Centre of University of Alberta and divided into four groups. Boiled and fried eggs were prepared as described in Nimalaratne *et al*. (Nimalaratne *et al*., 2011) and yolks were separated from whites. To prepare scrambled eggs, yolks were separated and combined and stirred on a pre-heated (205 °C) frying pan (model SK200TY non-stick frying pan, Black & Decker Canada Inc., Brockville, ON) by stirring yolks for 1 min. Fresh yolks were separated from whites, pooled and used as the control sample. Cooked and raw egg yolks were frozen immediately at -20 °C and then subjected to freeze drying in containers covered with aluminum foil to protect from light. Freeze-dried egg yolks were ground using a mortar and pestle to obtain a fine homogenized powder. Samples were stored at -80 °C until further analysis.

5.2.3. Step-wise Extraction of Egg Yolk Antioxidants

The complete extraction procedure is illustrated in Figure 5.1. Egg yolk was first separated into lipophilic and hydrophilic fractions. For this, total lipids were extracted from the yolk using the method described by Furusawa *et al* (Furusawa, Ozaki, Nakamura, Morita, & Okazaki, 1999). Briefly, 5 g of freeze-dried egg yolk was homogenized with 40 mL *n*-hexane and 20 mL acetonitrile using an IKA T25 digital Ultra-Turrax homogenizer (IKA® Works, Wilmington,

NC, USA) followed by centrifugation at 3500 rpm for 5 min. The supernatant was poured into the separatory funnel and the top hexane layer was collected into a conical flask. The acetonitrile layer contained some amount of carotenoids, it was collected separately. The extraction was repeated one more time. The combined supernatants were transferred into a pre-weighed round bottom flask and concentrated using rotary evaporator under vacuum at room temperature. After flushing with nitrogen to remove the remaining hexane, it was weighed to determine the amount of lipids. The acetonitrile layers were also evaporated under nitrogen, reconstituted in 0.5 mL methanol and kept at -20 °C until analysis. The dried lipids were re-dissolved in 10 mL of 100% acetone and placed in -80°C for 3-4 hrs to solidify lipids. Then, the samples were vacuum filtered into an Erlenmeyer flask using a sintered glass funnel in a freezer compartment at -20°C to remove the crystallized lipids (Rodriguez-Amaya, 2003). The white crystalized lipid collected in the sintered glass funnel was re-dissolved in 10 mL acetone, placed at -80°C for 3-4 hrs and repeat the filtration another two times. The supernatants containing carotenoids were evaporated under N_2 and re-constituted in 1 mL methanol and combined with the previous carotenoid extract in acetonitrile. The lipids collected was then completely transferred using hexane into a conical flask and used to extract vitamin E. Around 40 mL of ethanolic potassium hydroxide (1:1 ethanol:KOH v/v) was added into the conical flask, mixed well and allowed for saponification at room temperature for 12-16 hrs (Surai, 2000). Then 40 mL of n-hexane was added and allowed to stand for 1 hr at room temperature. The top hexane layer was collected, evaporated to dryness and reconstituted in 1.5 mL of methanol:TBME (2:1 v/v).

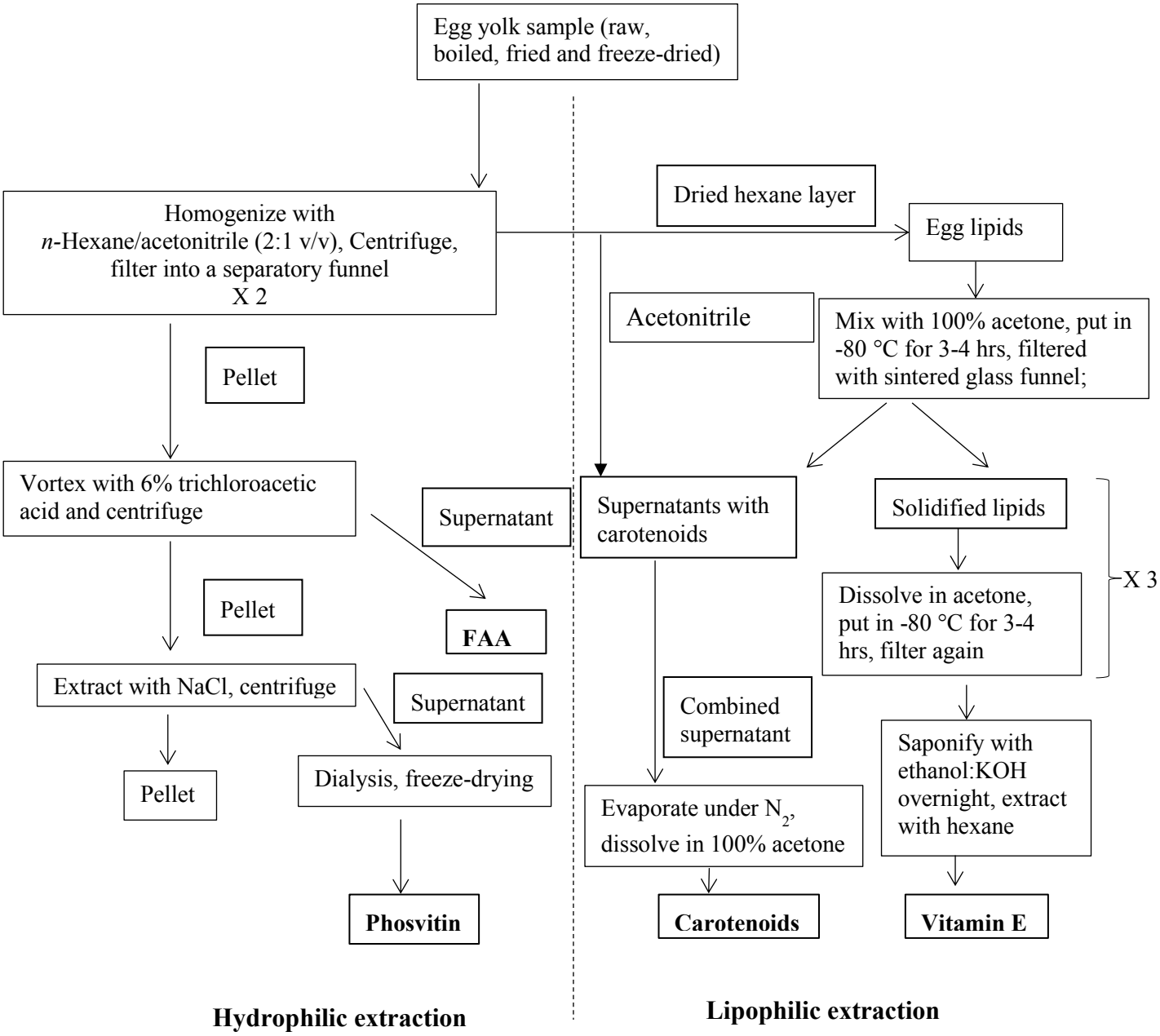

Figure 5.1: The flow diagram of stepwise extraction conditions to separate egg yolk antioxidants

The remaining pellet after lipid extraction was flushed with nitrogen until completely dried and used to extract hydrophilic antioxidants FAAs and phosvitin. For FAA extraction, the pellet was

homogenized with 10 mL of 6% trichloroacetic acid as described before (Nimalaratne *et al*., 2011). The residue obtained after amino acid extraction was used to extract phosvitin according to Ren & Wu (2014) with slight modifications. The residue was mixed with 50 mL of 10% NaCl and stirred overnight at 4 °C. The mixture was then subjected to 24 hr dialysis at 4 °C with 4-5 water changes followed by centrifugation at 10000 *g*, 4 °C for 25 min. The supernatant was freeze dried to obtain egg yolk phosvitin rich fraction. The four fractions with carotenoids, vitamin E, FAAs and phosvitin were subjected to quantitation analysis and antioxidant assays. In order to compare with the quantities obtained by stepwise extractions, all four compounds mentioned above were directly extracted from freeze dried egg yolk and subjected to quantification.

5.2.4. Antioxidant Assays

5.2.4.1. Free Radical-Scavenging Activity on ABTS•+

The free radical scavenging capacity using the ABTS radical cation ($ABTS^{\bullet+}$) was performed using the decolorization assay described by Madhujith *et al*.(Madhujith, Izydorczyk, & Shahidi, 2006) with slight modifications as described before (Nimalaratne *et al*., 2011)

5.2.4.2. Oxygen Radical Absorbance Capacity (ORAC)

The lipophilic (L-ORAC) and hydrophilic (H-ORAC) assays were performed according to Prior *et al*. (Prior *et al*., 2003). For the H-ORAC, a series of dilutions of the extracted samples and the Trolox standard solutions were prepared using 75 mM phosphate buffer at pH 7.4. For L-ORAC, 50% acetone was used to prepare the dilution series of samples and Trolox standard solutions. The assays were conducted using the method previously described (Nimalaratne *et al*., 2011) and ORAC values were calculated by dividing the slope of sample regression curve by the slope of

Trolox regression curve and the values were expressed as micromoles of Trolox equivalent (TE) per mg of yolk (μmol TE/mg).

5.2.5. Analysis of Carotenoid and Vitamin E Content using UFLC chromatography

Samples were analyzed using a Shimadzu UFLC-XR system (Shimadzu, Kyoto, Japan) equipped with a degasser, binary pump, autosampler, column oven, a diode array detector, and a communication bus module. The autosampler temperature was maintained at 15 °C during analyses. The separation of analytes was performed on a polymeric YMC C_{30} reversed-phase column (100 mm x 2.0 mm I.D. and 3 μm particle size, YMC America, Allentown, PA, USA) operated at 21°C. The injection volume was 3 uL. The composition of mobile phase A was methanol:water (90:10, v/v); mobile phase B consisted of TBME:methanol (80:20, v/v). The gradient program was 0−8 min (8−40% B), 8−13 min (40−100% B), 13−14.5 min (100% B), 14.5-14.6 min (8% B) and the flow rate was 0.3 mL/min. The detection was performed at 285 nm for vitamin E and 450 nm for carotenoids.

5.2.6. Analysis of Phosvitin Using Gel Filtration Chromatography

Phosvitin sample solutions were prepared in 0.1 M sodium phosphate buffer at 1 mg/mL concentration and the quantification was carried out using the conditions described before (Ren & Wu, 2014).

5.2.7. HPLC Analysis of FAAs

HPLC analysis of FAAs was carried out as described previously (Sedgwick *et al*., 1991) using ethanolamine and β-amino-n-butyric acid as the internal standards.

5.2.8. Statistical analysis

All data were analyzed by Analysis of variance (ANOVA) followed by Duncan's multiple range test using Statistical Analysis System Software (SAS version 9.0, SAS Institute, Cary, NC). Significance of differences was defined at the 5% level ($p < 0.05$)

5.3. Results and Discussion

The average lipid contents extracted from 5 g of freeze dried egg yolk were 2.24 ± 0.03, 2.62 ± 0.05 and 2.51 ± 0.2 for raw, fried and boiled samples respectively and showed a good agreement with previous studies (Furusawa *et al*., 1999; Nielsen, 1998). Since carotenoids can be subjected to degradation in the presence of light and oxidation (Schieber & Carle, 2008; Boon, McClements, Weiss, & Decker, 2010), dim light conditions and nitrogen purged solvents were used where applicable.

5.3.1. Quantification of free amino acids, phosvitin, vitamin E and carotenoids in different fractions of egg yolk

A stepwise method was developed to extract lutein, zeaxanthin, vitamin E, free amino acids and phosvitin from egg yolk and the quantities obtained by direct extraction in comparison to the stepwise extraction for raw egg yolk are shown in Table 5.1. The quantity of FAAs in raw egg yolk was 9078 ± 479 μg/g yolk. This is lower than the value obtained by direct extraction from the same sample of raw eggs, which was 10945 μg/g yolk (Table 5.1).This difference was possibly due to the losses that may have occurred during the lipid extraction. Nevertheless, vitamin E and carotenoids obtained after stepwise extraction, were not different from the quantities in the direct extracts (Table 5.1).

Table 5.1: Quantities of antioxidant compounds obtained after stepwise extraction and direct extraction

Antioxidant compound	Quantity	
	Step-wise extraction [a]	Direct extraction [b]
Free amino acids (µg/g)	9078 ± 479	10945 ± 271
Lutein (µg/100 g)	824.0 ± 45.2	779.4 ± 13.3
Zeaxanthin (µg/100 g)	392.5 ± 6.7	332.3 ± 14.6
Vitamin E (mg/ 100 g)	5.8 ± 0.6	6.4 ± 0.7

[a] – mean ± SD of three replicates; [b] – mean ± SD of two replicates

The quantities of lutein, zeaxanthin, vitamin E and FAAs obtained for fractions from different yolk samples were shown in Figure 5.2. Boiling and frying resulted in lower quantities of free amino acids compared to uncooked sample, but losses during scrambling was not significantly different. Egg yolk contains approximately 4% phosvitin based on dry weight (Powrie & Nakai, 1986). In this study, the content of phosvitin in raw yolk was calculated as 5.4% which is similar to previous data obtained from gel-filtration HPLC (Lei & Wu, 2012). These values obtained under HPLC are higher than those calculated based on nitrogen and phosphorus contents (Anton, Castellani, & Guérin-Dubiard, 2007; Castellani, Martinet, David-Briand, Guérin-Dubiard, & Anton, 2003). This may be due to contaminants in the HPLC sample, which resulted in overestimation of phosvitin concentration. The amounts of phosvitin in cooked samples were lower than the raw yolk (Figure 5.2). Vitamin E content in the extracted fraction from raw eggs was 5.8 ± 0.5 mg/100 g yolk. Vitamin E in the poultry feed can be partially transferred into the egg yolk (Surai, 2000), which might be the reason for the wide range of vitamin E levels

reported in the literature from 0.6 - 7.0 mg/100 g dry yolk (Murcia, Martinez-Tome, Cerro, Sotillo, & Ramirez, 1999). Although scrambled egg yolk contained significantly lower contents of vitamin E compared to raw yolk, boiling and frying did not cause significant reductions. Murcia *et al*. (Murcia, Martinez-Tome`, Cerro, Sotillo, & Ramirez, 1999) also investigated the losses of vitamin E in egg yolk during cooking and found microwave cooking and making omelets caused greater reductions compared to boiling which agrees with our results.

Carotenoids in egg yolk also depend on the amount and composition present in poultry feed (Schlatterer & Breithaupt, 2006). Lutein and zeaxanthin were the major carotenoids present in the extracted egg yolk fraction. In raw egg yolk, 824 ± 45 µg/100 g of lutein and 392 ± 6 µg/100 g zeaxanthin were present. Scrambling of eggs caused the greater reductions in lutein and zeaxanthin (Figure 5.2C). Frying and boiling caused 13% and 23 % losses of lutein from fried and boiled samples respectively. Previous reports on effect of cooking on egg carotenoids are controversial. Surai *et al*. (Surai, MacPherson, Speake, & Sparks, 2000) found that the concentration of lutein in "designer eggs" does not decrease during boiling. In contrast, Schlatterer & Breithaupt found that the losses of lutein were 19 ± 15% and zeaxanthin 15 ± 11% after boiling of eggs, which are similar to our observations. They estimated that the degradation of xanthophylls during household cooking of eggs can be roughly between 10 and 20% (Schlatterer & Breithaupt, 2006).

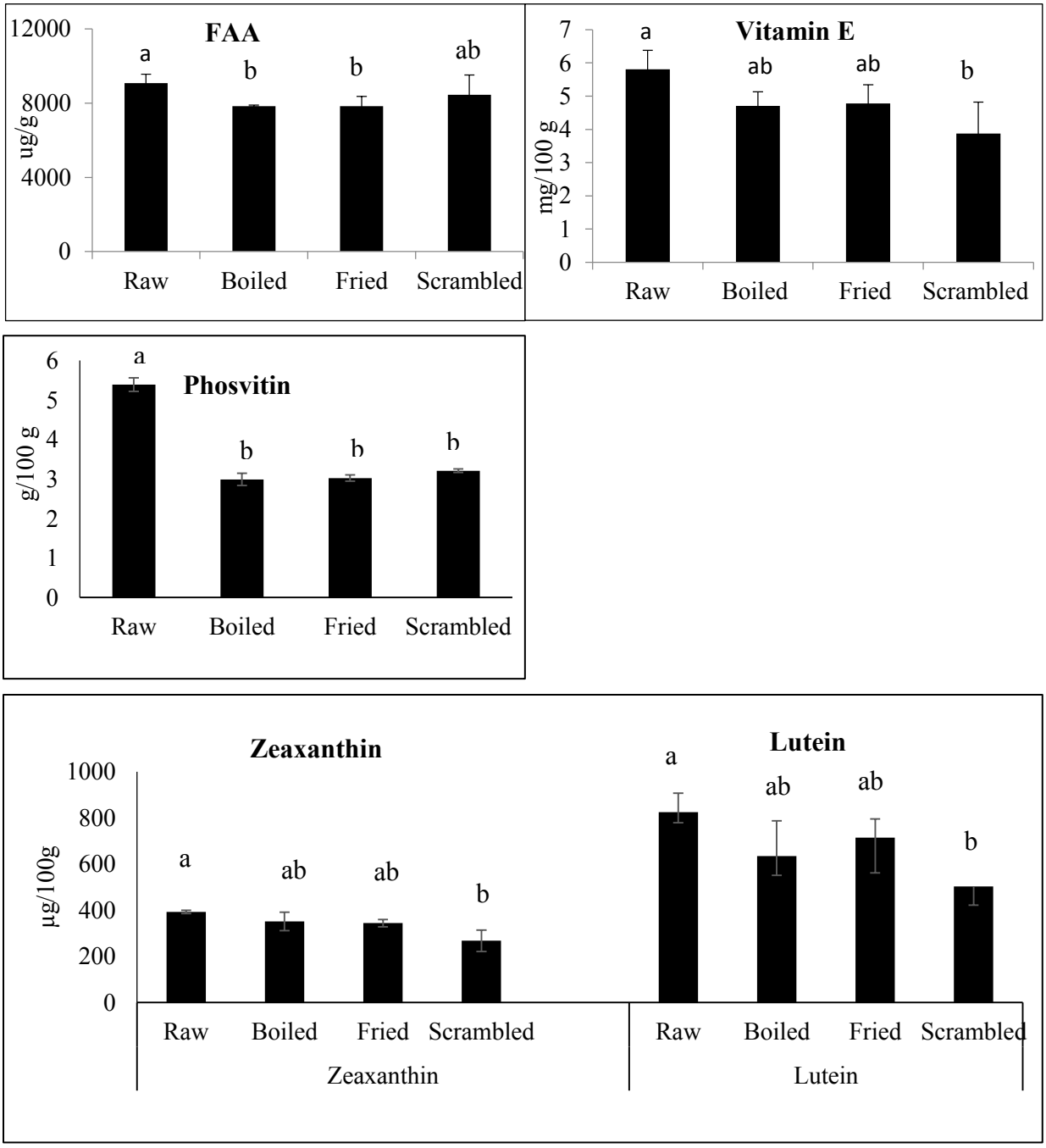

Figure 5.2: Effect of cooking on free amino acids, vitamin E, phosvitin and yolk carotenoids. [a-b]

Different letters denote significant differences at $P<0.05$

5.3.2. Antioxidant activity of different fractions

The FAA fraction of raw yolk has the highest ORAC activity of 115.9 ± 14.8 µmol TE/ g freeze dried yolk (Table 5.2). It was approximately, 67% of the total ORAC activity of 172 µmol TE/ g. ORAC activity of phosvitin and carotenoids were similar to each other while vitamin E showed the lowest activity. Total free amino acid content have been reported to correlated with radical scavenging activity (Pérez, Iglesias, Pueyo, Gonzalez, & de Lorenzo, 2007). More specifically, amino acids such as tryptophan, tyrosine, histidine, methionine, valine etc are known to have high antioxidant activity (Clausen, Skibsted, & Stagsted, 2009; Katayama & Mine, 2007).

Table 5.2: Antioxidant activity of fractions obtained from stepwise extraction of raw egg yolk

Antioxidant compound	ORAC (umol TE/ g freeze dried yolk)		ABTS (umol TE/ g freeze dried yolk)
	H-ORAC	L-ORAC	
Free amino acids	115.9 ± 14.8	-	35.5 ± 3.5
Carotenoids	-	24.7 ± 3.0	-
Phosvitin	25.3 ± 2.5	-	-
Vitamin E	-	6.5 ± 1.6	-

Lutein and zeaxanthin are the main carotenoids in egg yolk and are known to possess radical scavenging activity (Müller, Fröhlich, & Böhm, 2011; Stahl & Sies, 2003). Vitamin E is also a well-known antioxidant and has been extensively studied on the effect of supplementing through hen's diet on the antioxidant system yolk and developing embryo (Meluzzi *et al*., 2000; Surai,

2000). Both lutein and vitamin E have been reported to have high ORAC activity (Naguib, 1998). In addition to their potential health benefits, carotenoids and vitamin E play a role in protecting other egg yolk nutrients from oxidation. For example, when used in combination, vitamin E and lutein significantly suppressed the formation of malondialdehyde in DHA-enriched eggs as a result of Fe-stimulated lipid oxidation (Surai *et al*., 2000). Similarly, supplementing with α-tocopherol improved the stability of cholesterol and carotenoids egg lipids subjected to nitric oxide-initiated oxidation (Lai, Gray, Chen, & Grulke, 1996).

Egg yolk phosvitin is a phosphorylated protein (Byrne *et al*., 1984) and known to have metal chelating ability and antioxidant activity (Castellani, Guérin-Dubiard, David-Briand, & Anton, 2004; Grizzuti & Perlmann, 1973; Lu & Baker, 1986). Radical scavenging activity by ABTS assay did not responded well to yolk fractions except for FAA. ABTS values for the free amino acids also showed a similar trend to the ORAC activity with the values of 35.5 ± 3.5, 25.3 ± 3.6, 30.4 ± 5.5, 21.49 ± 1.8 umol TE/ g freeze dried yolk for the raw, boiled, fried and scrambled egg yolk respectively.

In general, cooking reduced the ORAC activity (Figure 5.3). The reductions of the activity were correlated with the quantitative losses except for the free amino acid fraction in the scrambled eggs. The FAA content of scrambles eggs was were not different with the other samples, however showed a lower ORAC value. This could be attributed to the factor that amino acid composition is more related to the antioxidant activity rather than the total content (Pownall, Udenigwe, & Aluko, 2010). Among three cooking methods, scrambling seems to be the most deleterious except in case of phosvitin (Figure 5.3). Preparation of scrambled eggs involves higher temperature (205 °C frying pan) and increased exposure to oxygen while stirring. This might provoke the oxidation/degradation of sensitive antioxidants such as FAA, carotenoids and

vitamin E. In case of phosvitin, which is usually highly resistance to processing conditions, scrambling may generate PPP and improve antioxidant activity. However, the activity is lower than the raw egg yolk phosvitin extract. Since the phosvitin fraction is a crude extract of phosvitin, other compounds present in that may contributed to the ORAC activity and degraded upon scrambling.

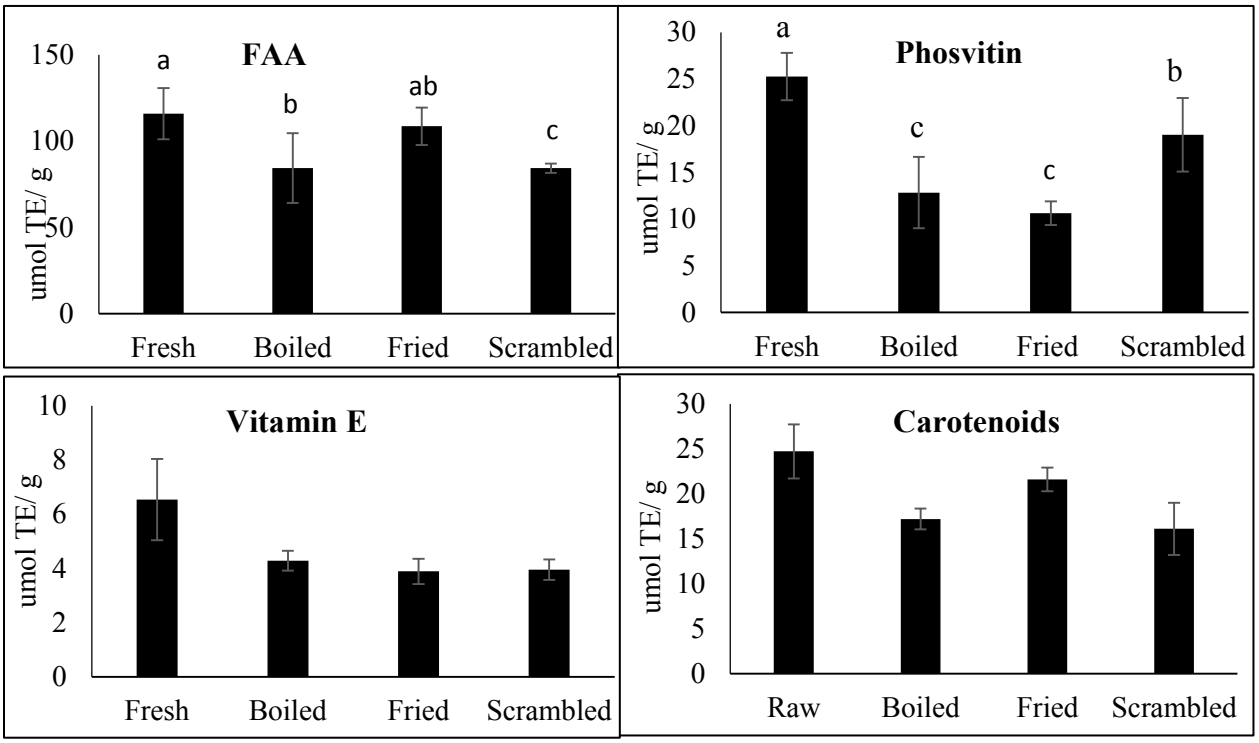

Figure 5.3: ORAC activity of free amino acids, phosvitin, vitamin E and carotenoids from egg yolk as affected by cooking methods. [a-c] Different letters denote significant differences at $P<0.05$

In summary, the antioxidant compounds in egg yolk were extracted in a stepwise process and the antioxidant activity of each fraction was analyzed. Approximately, two thirds of the ORAC activity is from free amino acids. In addition, carotenoids and phosvitin and vitamin E also contribute to the total antioxidant activity. All cooking treatments reduced the antioxidant activity. Our further studies are to determine the effect of gastrointestinal digestion on the total antioxidant activity of egg yolk compounds.

CHAPTER 6 - Effects of Storage and Cooking on the Antioxidant Capacity of Laying Hen Eggs[3]

[3]A version of this chapter has been submitted for publication. Nimalaratne, C., Schieber, A., & Wu, J. Effects of Storage and Cooking on the Antioxidant Capacity of Laying Hen Eggs. *Food Chemistry*.

6.1. Introduction

Chicken eggs are economically affordable nutritious food commodity containing important macronutrients such as highly digestible proteins and lipids, as well as micronutrients such as vitamins and minerals. Eggs are also viewed as an efficient vehicle for delivering various nutrients, for example omega-3 fatty acids, vitamin E, selenium, and lutein which are thought to provide additional protection against certain disease conditions (Sim, 1998). Eggs enriched with these nutrients are referred to as 'designer eggs' which can be obtained through formulating hens' feed (Surai & Sparks, 2001). The concept of designer eggs fits nicely with the increasing needs of health conscious consumers, with omega-3 enriched eggs being most popular (Sim & Sunwoo, 2002). Egg constituents also impart various biological activities including antimicrobial, antioxidant, antihypertensive and anticancer activities, immunomodulatory and antiadhesive properties, which may be beneficial beyond the basic nutrition (Kovacs-Nolan, Phillips, & Mine, 2005). Among various bioactivities, the antioxidant activity of egg-derived compounds has attracted great attention due to the possible roles of antioxidants against various chronic diseases including heart diseases (Dávalos, Miguel, Bartolomé, & López-Fandiño, 2004; Katayama, Ishikawa, Fan, & Mine, 2007). Recently, we found that two aromatic amino acids, tryptophan and tyrosine, contribute to the antioxidant properties of egg yolk (Nimalaratne, Lopes-Lutz, Schieber, & Wu, 2011). Egg proteins and derived peptides, phospholipids, vitamin E, carotenoids and phosvitin are other compounds that contribute to the total antioxidant activity of egg yolk (Hargitai *et al*., 2006; Katayama, Xu, Fan, & Mine, 2006; Mohiti-Asli, Shariatmadari, Lotfollahian, & Mazuji, 2008).

However, the antioxidant properties of egg yolk may vary depending on several factors for instance egg variety, processing and storage conditions. In Canada, approximately 70% of the total eggs produced are consumed as table eggs, which are stored at refrigerated temperature with an approximate shelf life of one month, while the rest is processed into liquid, frozen or dried products (Agriculture and Agri-Food Canada, 2013). It is known that industrial processing techniques such as spray-drying can result in increased lipid oxidation and loss of antioxidant capacity in eggs (Galobart, Barroeta, Baucells, & Guardiola, 2001; Lai, Gray, Buckley, & Kelly, 1995; Morgan & Armstrong, 1992). Table eggs are usually subjected to domestic cooking methods such as frying, boiling, scrambling etc. which are also known to reduce the antioxidant properties and the content of free amino acids (Nimalaratne *et al*., 2011), and increase lipid oxidation of eggs, especially in n-3 PUFA enriched eggs (Cortinas, Galobart, Barroeta, Baucells, & Grashorn, 2003; Ren, Perez, Zuidhof, Renema, & Wu, 2013). Lipid oxidation occurs during storage, especially at elevated temperatures (Mohiti-Asli *et al*., 2008), and is more pronounced in n-3 PUFA enriched eggs due mainly to the presence of the unsaturated bonds (Hayat, Cherian, Pasha, Khattak, & Jabbar, 2010; Ren *et al*., 2013). Most available data on lipid oxidation or oxidative stability are focused on dried egg products and omega-3 enriched eggs, whereas information on the antioxidant activity of table eggs during retail storage and cooking is limited. Given the fact that free aromatic amino acids and carotenoids are the major contributors to the antioxidant capacity of egg yolk, the main objective of this study was to evaluate the effects of simulated retail storage conditions and cooking on the antioxidant capacity, the contents of free amino acids and carotenoids, and lipid oxidation. To compare the antioxidant capacity of different eggs, four different types of eggs were used in the study.

6.2. Materials and methods

6.2.1. Chemicals

Lutein (xanthophyll from marigold), zeaxanthin, trichloroacetic acid, 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2-thiobarbituric acid (TBA), and anhydrous monobasic sodium phosphate, were obtained from Sigma–Aldrich (Oakville, ON, Canada). Light petroleum ether, methanol, acetone, ethyl acetate, TBME (*tert*-butyl methyl ether) and HPLC-grade water were purchased from Fisher Scientific (Ottawa, ON, Canada). Fluorescein disodium and trolox were obtained from Acros Organics (Morris Plains, NJ, USA).

6.2.2. Types of Eggs

Four types of fresh shell eggs (n=72 each) were used in this study. Normal table eggs and eggs from two heritage chicken breeds (white leghorns and brown leghorns) were obtained from Poultry Research Centre of the University of Alberta (Edmonton, AB, Canada). Heritage chicken breeds are characterized by an original genetic line; they are not selected based on egg laying or quality performances. Eggs enriched in lutein and omega-3 fatty acids were purchased from a local supermarket (Edmonton, AB, Canada). Eggs were stored under normal retail storage conditions, i.e. at 4 °C in packages for 35 days and analyzed on a weekly basis.

6.2.3. Cooking of Eggs

A set of twelve eggs was taken from each type and divided into three groups for boiling, frying and raw egg yolks. For boiling, whole shell eggs were placed in a saucepan as a single layer, with water up to 1-2 inches above the eggs, and boiled for 10 min. The eggs were then placed under running tap water for 5 min and, peeled, and yolks were separated from whites. Fried eggs

were prepared using a frying pan (model SK200TY non-stick frying pan, Black & and Decker Canada Inc., Brockville, ON, Canada) preheated to 205 °C. Eggs were fried for 6 min (3 min each side) and the yolks were separated from whites. Raw egg yolks were used as a control. To prepare raw yolk samples, egg yolks were manually separated from whites and wiped with a filter paper to remove adhered albumins. Cooked and raw egg yolks were pooled and subjected to freeze drying in containers covered with aluminum foil to protect samples from light induced oxidation. Dim light conditions were used throughout the cooking period. Freeze-dried samples were ground to obtain a fine powder. The processed samples were stored in the dark at -20 °C in airtight sealed plastic containers until analysis. Cooking and analysis of samples were carried out during the storage period on days 1, 7, 14, 21, 28, 35.

6.2.4. Extraction and Analysis of Free Amino Acids

Free amino acids were extracted using the method described by Ohkubo, Sawaguchi, Hamatsu, and Matsubara, (2006). Approximately 150 mg of freeze-dried egg yolk was extracted with 1 mL of 6% trichloroacetic acid by vortexing followed by centrifugation at 6000 *g* for 20 min using a bench top centrifuge (Centrifuge 5418, Eppendorf, Hamburg, Germany). The supernatant was filtered with 0.45 μm nylon syringe filter (Mandel Scientific Corp., Guelph, ON, Canada) and used for HPLC analysis. Free amino acids were determined as described by others previously (Sedgwick, Fenton, & Thompson, 1991) using ethanolamine and β-amino-n-butyric acid as the internal standards.

6.2.5. Extraction and Analysis of Carotenoids

Yolk samples were extracted according to Schlatterer and Breithaupt (2006) with minor modifications as described previously (Nimalaratne, Lopes-Lutz, Schieber, & Wu, 2012).

Approximately 0.5 g of freeze dried egg yolk powder was extracted three times (3 mL each) using a ternary solvent mixture (methanol: ethyl acetate: petroleum ether, 1:1:1, v/v/v) with 0.1% BHT and the combined supernatants were evaporated under nitrogen gas. The residue was dissolved in 2 mL of TBME:methanol (3:1, v/v). These samples were filtered through 0.45 µm nylon membrane filter and analyzed by UFLC. Duplicate extractions were performed and the samples were protected from light during the extraction and analysis.

Samples were analyzed using a C_{30} reversed-phase column, 100 mm x 2.0 mm I.D. and 3 µm particle size (YMC America, Allentown, PA, USA) operated at 21°C. Analyses were performed on a Shimadzu UFLC-XR system (Shimadzu, Kyoto, Japan) equipped with a diode array detector (Model SPD-M20A). The injection volume was 3 µL. A binary solvent system consisting of mobile phase A (methanol:water, 90:10, v/v) and mobile phase B (TBME:methanol, 80:20, v/v) was used with the following gradient: 0–8 min (8 − 40% B), 8–13 min (40 − 100% B), 13–14.5 min (100% B), 14.5-14.6 min (8% B). . The flow rate was 0.3 mL/min and the carotenoids were detected at 450 nm.

6.2.6. Oxygen Radical Absorbance Capacity (ORAC)

Approximately 50 mg of freeze dried egg yolk sample was extracted with 0.5 mL of acidified (pH 1.5) 80% methanol in a microcentrifuge tube using a vortex mixer and centrifuged at 6000 *g* for 10 min (Centrifuge 5418, Eppendorf, Hamburg, Germany). The supernatant was evaporated under vacuum at 30 °C using a vacuum rotary evaporator and reconstituted with 200 µL of methanol. All samples were filtered with a 0.22 µm nylon syringe filter and a series of dilutions were prepared for ORAC assay. The assay was conducted using a previously described method (Nimalaratne *et al*., 2011) and ORAC value were calculated by dividing the slope of sample

regression curve by the slope of Trolox regression curve. The results were expressed as micromoles of Trolox equivalent (TE) per mg of yolk (μmol TE/mg).

6.2.7. Spectrophotometric Determination of Thiobarbituric Acid Reactive Substances (TBARs)

The TBARs test was performed according to the method reported by Ren *et al*. (2013) with minor modifications. Briefly, yolk samples (0.6 g) were homogenized with 2.5 mL of 1.15% KCl using an IKA T25 digital Ultra-Turrax homogenizer (IKA® Works, Wilmington, NC, USA). Subsequently, 1 mL of the homogenate was combined with 125 μL of 80 mM Tris/ maleate buffer solution and 625 μL of TCA-TBA-HCl solution, mixed rapidly and incubated in the dark for 30 min at 70 °C. The samples were centrifuged at 16000 *g* for 5 min and the supernatants pipetted into a 96-well plate (Costar #9017, Corning, Inc., NY, USA). Absorbance values were recorded at 532 nm using a (SpectraMax M3 multi-mode microplate reader, Molecular device, Sunnyvale, CA, USA). The results were calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and expressed as mg of malondialdehyde (MDA) per kg of sample (Ren *et al*., 2013).

6.2.8. Statistical Analysis

All data obtained for free amino acid content, ORAC activity, carotenoid content and TBARs were individually analysed with factorial ANOVA to study the influence of three cooking treatments on four types of eggs at six different time points over 35 days of storage period using the PROC MIXED procedure of Statistical Analysis System Software (SAS version 9.3, SAS Institute, Cary, NC). Least square means (ls means) were calculated and separated using Tukey's adjustment test with the significant level at $P < 0.05$.

6.3. Results and discussion

6.3.1. Effect of Storage and Cooking on Free Amino Acid Content

The average total free amino content of the raw egg yolk from different types of eggs ranged from 8209.4 to 9674.5 µg/g yolk (Table 6.1). Total amino acid contents of heritage breeds were slightly lower but not significantly different from the normal table eggs and n-3/lutein enriched eggs.

Table 6.1. Free amino acid content (µg/g sample) in different types of cooked eggs*

Treatment	Normal	n-3/ lutein enriched	HB	HW
Week 1				
Raw	9674[a]	9242[a]	8209[a]	8705[a]
Boiled	7771[b]	7222[b]	7535[ab]	6707[b]
Fried	6900[b]	7014[b]	6172[b]	6516[bc]
Week 6				
Raw	7426[b]	7930[ab]	7558[ab]	7557[ab]
Boiled	5740[c]	7731[b]	7160[b]	6265[bc]
Fried	5226[c]	6974[b]	6968[b]	5890[c]

* Each value is the average of four replicates. Abbreviations: HB, Heritage brown leghorn; HW, Heritage white leghorn; n-3/L, n-3 PUFA and lutein enriched. [a-c] Means within a column with no common superscripts differ at $P < 0.05$.

During storage, free amino acid contents were stable with minor fluctuations in the quantity, (Figure 6.1A). Except for normal eggs, the total free amino acid content of all types of eggs remained unchanged after six weeks of storage. With regard to the individual amino acids, the

aromatic amino acids tryptophan (Trp) and tyrosine (Tyr) which were reported to have high antioxidant activity (Herraiz & Galisteo, 2004; Huang, Majumder, & Wu, 2010; Tsopmo *et al*., 2009) were found to be highest (Tyr 676.7 ± 42.2 and Trp 97.9 µg/g yolk) in fresh n-3/lutein enriched eggs, while the Tyr content was significantly reduced (587.4 ± 12.4 µg/g yolk) during storage (Table 6.2). This may possibly contribute to the declined ORAC activity (from 161.4 to 114.5 µmol TE/g) in n-3/lutein enriched eggs during storage (Table 6.1). The Trp and Tyr contents of other types of eggs were not affected by storage.

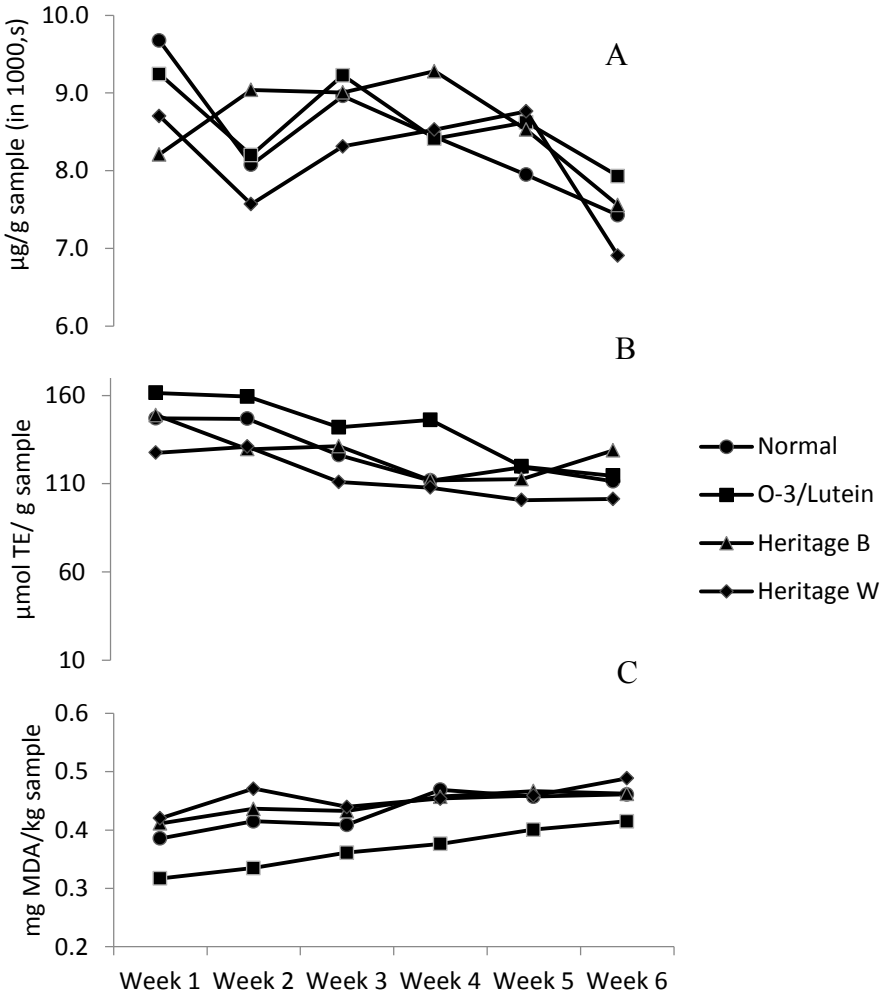

Figure 6.1 Effect of storage on the total content of free amino acid (A), ORAC values (B), and MDA Content (TBARs, C)

Table 6.2. Free amino acid content of the raw yolk samples (µg/g) [*]

Amino acid	Normal table eggs		n-3 PUFA/lutein enriched		Heritage brown leghorn		Heritage white leghorn	
	Week 1	Week 6	Week 1	Week 6	Week 1	Week 6	Week 1	Week 6
Aspartic	523.2 ± 79.5^{a}	423.4 ± 13.6^{ab}	479.9 ± 13.5^{ab}	488.4 ± 6.2^{ab}	358.7 ± 49.1^{b}	432.9 ± 0.0^{ab}	455.6 ± 47.6^{ab}	417.4 ± 2.7^{ab}
Glutamic	1591.3 ± 231.2^{a}	1057.0 ± 47.6^{b}	1245.4 ± 45.9^{ab}	1158.6 ± 38.3^{ab}	1120.2 ± 168.0^{b}	1154.7 ± 47.9^{b}	1292.8 ± 78.4^{ab}	998.1 ± 20.6^{b}
Asparagine	396.9 ± 64.3^{a}	265.2 ± 3.8^{b}	298.4 ± 2.1^{ab}	270.8 ± 5.9^{b}	286.3 ± 46.0^{ab}	276.9 ± 12.1^{b}	325.8 ± 23.8^{ab}	253.3 ± 5.0^{b}
Serine	694.6 ± 90.5^{a}	471.8 ± 6.4^{b}	592.2 ± 2.0^{ab}	501.1 ± 1.7^{b}	491.7 ± 67.8^{b}	457.7 ± 8.6^{b}	563.6 ± 65.3^{ab}	442.5 ± 6.9^{b}
Glutamine	503.9 ± 48.4^{a}	337.2 ± 8.4^{ab}	445.8 ± 5.0^{ab}	281.0 ± 93.1^{b}	448.4 ± 62.8^{ab}	377.1 ± 9.2^{ab}	491.6 ± 38.7^{a}	346.7 ± 26.7^{ab}
Histidine	179.5 ± 17.9^{a}	149.1 ±4.6 ^{a}	165.2 ± 2.0 ^{a}	110.8 ± 53.3 ^{a}	158.1 ± 23.7 ^{a}	150.8 ± 6.7 ^{a}	154.2 ± 14.4 ^{a}	138.5 ± 3.3 ^{a}
Glycine	223.4 ± 16.1 ^{a}	186.3 ± 2.6 ^{a}	225.9 ± 6.4 ^{a}	237.3 ± 33.1 ^{a}	198.9 ± 25.5 ^{a}	192.5 ± 10.6 ^{a}	220.6 ± 3.0 ^{a}	181.3 ± 6.2 ^{a}
Threonine	524.2 ± 25.1 ^{a}	425.0 ± 1.5 ^{a}	515.5 ± 7.2 ^{a}	350.7 ± 191.1 ^{a}	497.5 ± 67.7 ^{a}	446.1 ± 19.7 ^{a}	538.3 ± 0.7 ^{a}	411.6 ± 29.5 ^{a}
Arginine	649.8 ± 10.3^{bc}	580.9 ± 6.6^{c}	776.6 ± 21.1^{a}	631.6 ± 27.4^{bc}	636.3 ± 48.4^{bc}	580.8 ± 33.9^{c}	709.1 ± 14.3^{ab}	568.3 ± 22.4^{c}
Alanine	311.1 ± 0.2 ^{a}	177.7 ± 78.4 ^{a}	319.7 ± 16.2 ^{a}	271.6 ± 6.2 ^{a}	274.5 ± 6.8 ^{a}	249.6 ± 10.6 ^{a}	310.2 ± 5.2 ^{a}	177.9 ± 79.4 ^{a}
Tyrosine	533.5 ± 2.0^{b}	541.1 ± 2.2 ^{b}	676.7 ± 42.2 ^{a}	587.4 ± 12.4 ^{b}	528.0 ± 5.9 ^{b}	585.7 ± 21.3 ^{b}	544.9 ± 11.6 ^{b}	522.3 ± 26.9 ^{b}
Tryptophan	85.9 ± 17.0 ^{a}	89.3 ± 1.4 ^{a}	97.9 ± 12.7 ^{a}	94.6 ± 5.1 ^{a}	74.0 ± 1.7 ^{a}	80.7 ± 8.7 ^{a}	78.8 ± 6.8 ^{a}	81.5 ± 0.4 ^{a}
Methionine	238.5 ± 43.3^{b}	190.9 ± 1.1 ^{b}	245.5 ± 18.0 ^{b}	533.9 ± 176.0 ^{a}	216.4 ± 13.7 ^{b}	141.2 ± 80.7 ^{b}	202.7 ± 10.4 ^{b}	174.0 ± 3.5 ^{b}
Valine	541.1 ± 85.9 ^{a}	406.0 ± 5.5 ^{a}	527.3 ± 27.9 ^{a}	106.8 ± 151.0^{b}	463.2 ± 18.5 ^{a}	316.3 ± 84.3^{ab}	440.3 ± 28.2 ^{a}	350.5 ± 16.7^{ab}
Phenylalanine	458.5 ± 81.4 ^{a}	408.6 ± 8.1 ^{a}	477.1 ± 43.3 ^{a}	440.2 ± 14.1 ^{a}	391.8 ± 20.1 ^{a}	398.6 ± 3.5 ^{a}	379.8 ± 27.3 ^{a}	342.9 ± 28.3 ^{a}
Isoleucine	420.4 ± 67.6^{a}	344.8 ± 6.7^{ab}	417.5 ± 4.4a^{b}	389.6 ± 13.3^{ab}	423.6 ± 38.6^{a}	366.0 ± 1.5^{ab}	364.5 ± 21.3^{ab}	299.4 ± 26.1^{b}
Leucine	846.2 ± 121.5^{a}	657.7±11.3^{abc}	788.8 ± 11.9^{ab}	681.9 ± 29.9^{abc}	786.7 ± 65.4^{ab}	623.9 ± 10.0^{bc}	749.5 ± 43.8^{abc}	549.7 ± 49.0^{c}
Lysine	952.4 ± 129.6^{a}	713.9 ± 19.9^{ab}	947.0 ± 21.1^{a}	793.7 ± 57.7^{ab}	855.2 ± 80.9^{ab}	726.2 ± 0.6^{ab}	882.6 ± 53.7^{ab}	650.8 ± 32.7^{b}

[A] Results are means ± standard deviation (n=4). [a-c] values with different superscripts within a row indicate significant differences at $P < 0.05$.

Both cooking methods significantly reduced the quantity of free amino acids in all types of eggs, with no differences being observed between frying and boiling (Table 6.1). Cooking was reported to reduce free amino acids and antioxidant capacity in different food products. Jensen, Dort, and Eilertsen (2014) showed that antioxidant capacity and content of taurine in pork and beef were significantly reduced by household cooking. Steaming of ginseng (*Panax ginseng*) caused a reduction in most free amino acids, especially arginine (Arg), the most predominant amino acid in ginseng, which decreased significantly from 10.4 mg/g to 1.4 mg/g (Cho *et al*., 2008).

6.3.2. Effects of Storage and Cooking on Carotenoid Content

Lutein and zeaxanthin were the major carotenoids found in eggs. The carotenoid profile of eggs largely depends on the carotenoid content and composition of the hens' feed (Schlatterer & Breithaupt, 2006). The eggs obtained from Poultry Research Centre of University of Alberta did not contain synthetic carotenoids as feed additives, which explained the presence of natural carotenoids (lutein and zeaxanthin) as the only carotenoids. In n-3/lutein enriched eggs, the zeaxanthin content was below the limit of quantification. According to the label, one enriched egg contains 0.5 mg of lutein which might be the reason for low zeaxanthin levels. A previous study also reported that increasing dietary doses of lutein can interfere with the deposition of zeaxanthin in egg (Leeson & Caston, 2004). The total lutein content was 3385.1 μg/100 g in n-3/lutein enriched eggs, whereas it ranged from 1096 to 1347.0 μg/100 g in other eggs (Table 6.3). The zeaxanthin content in normal, HB and HW eggs were in the range of 496.4 to 665.1 μg/100 g which is in accordance with previously published data (Nimalaratne *et al*., 2012; Schlatterer & Breithaupt, 2006; Wenzel, Seuss-Baum, & Schlich, 2011). Lutein and zeaxanthin contents in eggs remained unaffected by the storage. Information on the stability of yolk

carotenoids in raw eggs during storage either refrigerated or at room temperature is limited. Barbosa, Gaspar, Calixto, and Agostinho (2011) reported that, after 14 days storage at refrigeration temperature, the total carotenoid content (measured spectrophotometrically) in raw eggs enriched with omega-3 fatty acids and carophyll (canthaxanthin preparation) decreased significantly, while at room temperature, losses were already observed after 7 days of storage. Cooking of eggs significantly reduced the quantity of carotenoids; similar results were reported by Nimalaratne *et al*., (2012) and Schlatterer and Breithaupt, (2006) who observed 6-18% of total carotenoid losses and 19 ± 15% losses of lutein after cooking of eggs, respectively.

Table 6.3. Lutein and zeaxanthin content (µg/100 g) in different types of eggs as affected by cooking and storage*

Treatment	Lutein				Zeaxanthin			
	Normal	n-3/L	HB	HW	Normal	n-3/L	HB	HW
Week 1								
Raw	1096.0^{a}	3385.1^{a}	1347.0^{a}	1302.3^{a}	665.1^{a}	tr	580.3^{a}	558.4^{a}
Boiled	934.8^{b}	3201.6^{b}	1068.5^{b}	1069.2^{bc}	564.6^{b}	tr	376.4^{b}	477.8^{b}
Fried	949.6^{b}	3075.9^{b}	1206.3^{b}	1204.0^{b}	507.8^{b}	tr	394.0^{b}	425.3^{b}
Week 6								
Raw	1083.5^{a}	3310.4^{a}	1282.1^{a}	1206.2^{ab}	603.1^{a}	tr	522.8^{a}	496.4^{a}
Boiled	859.3^{b}	2888.9^{c}	872.8^{b}	865.1^{d}	509.9^{b}	tr	425.1^{b}	407.5^{b}
Fried	862.8^{b}	2785.9^{c}	960.5^{b}	1023.2^{c}	468.8^{b}	tr	392.4^{b}	394.6^{b}

* Values were expressed as means of four replicates. $^{a-d}$ Means within a column with no common superscripts differ at $P < 0.05$. Heritage brown leghorn, HB; Heritage white leghorn, HW; n-3 PUFA and lutein enriched, n-3/L.

6.3.3. Effect of Storage and Cooking on ORAC Activity

Antioxidant activity measured as oxygen radical scavenging capacity was highest in n-3/lutein enriched eggs (161.4 µmol TE/g) followed by normal table eggs, heritage brown leghorn and heritage white leghorn eggs (Table 6.4). The ORAC activity was not different among the different types of eggs and remained unaffected by the storage after six weeks at 4 °C in all types of eggs. Similar to the free amino acid content in egg yolk, ORAC values showed a decreasing trend during storage (Figure 6.1). As reported previously (Nimalaratne *et al*., 2011; Remanan & Wu, 2014), both boiling and frying of eggs significantly reduced the ORAC activity irrespective of the type of egg (Table 6.4).

Table 6.4. Changes of ORAC content (µmol TE/g sample) in different types of cooked eggs during storage*

	Treatment	Normal	n-3/ lutein enriched	HB	HW
Week 1	Raw	147.1[a]	161.4[a]	148.9[a]	127.6[a]
	Boiled	74.8[b]	74.2[b]	61.7[b]	73.2[b]
	Fried	83.5[b]	71.9[b]	62.7[b]	77.1[b]
Week 6	Raw	111.3[a]	114.5[a]	128.8[a]	101.3[a]
	Boiled	41.2[b]	57.8[b]	56.7[b]	56.7[b]
	Fried	48.5[b]	41.7[b]	45.7[b]	45.7[b]

* Values were expressed as mean of eight replicates. [a-b] Values with different superscripts within a column indicate significant differences at $P < 0.05$

No differences were observed between frying and boiling treatments. The effect of cooking on antioxidant activity is dependent on the type of food and physicochemical changes during the cooking process. For example, cooking (such as microwave cooking, steaming, or boiling) increased the antioxidant activity of certain foods like carrots, spinach, mushrooms, asparagus, tomato, cabbage, red cabbage, etc but decreased it in steamed rice, corn grits, potato tubers, Jalapeño and Serrano peppers etc (Alvarez-Parrilla, de la Rosa, Amarowicz, & Shahidi, 2011; Halvorsen & Carlsen, 2006; Perla, Holm, & Jayanty, 2012). Release of bound and trapped antioxidants during cooking may enhance the total antioxidant capacity, whereas degradation, isomerization, or oxidation of antioxidants during heating reduces total activity. In egg yolk, the main compounds contributing to ORAC are free amino acids and carotenoids (Nimalaratne *et al*., 2011). These compounds are susceptible to heating and oxidation, but comparatively stable at low temperature storage and as a result, affect the total antioxidant activity.

6.3.4. Effects of Storage and Cooking on Thiobarbituric Acid Reactive Substances

Lipid oxidation of egg yolk, especially in omega-3 enriched eggs has been reported previously and found to increase with processing, storage and exposure to light (Galobart, Guardiola, Dutta, Codony, & Savage, 2002). During storage at 4 °C, MDA contents of all four types of eggs continuously increased (Figure 6.1) but were not significantly different from those of fresh raw eggs. MDA contents of fresh raw eggs ranged from 0.32 to 0.42 mg/kg sample (Table 6.5) while no differences were observed among four types of eggs. These results are in agreement with results published by Mohiti-Asli *et al*. (2008), who did not observe changes in MDA content in eggs stored at 4 °C for two weeks but found an increase at room temperature. Both boiling and frying increased the MDA content in all egg types and the increments were higher in aged eggs (Table 6.5). Cortinas *et al*. (2003) and Ren *et al*. (2013) reported that domestic cooking (frying,

scrambling and boiling) significantly increased MDA contents in eggs. Boiling and frying was not different with regard to the generation of MDA in fresh eggs. However, after six weeks of storage, frying seemed to produce higher amounts of MDA than boiling (Table 6.5). Ren *et al* also reported that frying led to a higher content of MDA than boiling, which was attributed to the high temperatures typically used in frying (Ren *et al*., 2013).

Table 6.5. Changes of malondialdehyde content (mg/kg sample) in different types of cooked eggs during storage

Treatment	Normal	% change	n-3/ lutein	% change	HB	% change	HW	% change
Week 1								
Raw	0.39[a]		0.32[a]		0.41[a]		0.42[a]	
Boiled	0.94[b]	141	0.89[b]	178	0.98[b]	139	0.98[b]	133
Fried	1.03[b]	164	1.01[b]	215	1.07[b]	161	1.08[b]	157
Week 6								
Raw	0.46[a]		0.41[a]		0.46[a]		0.49[a]	
Boiled	1.41[c]	206	1.57[c]	283	1.72[c]	274	1.75[c]	257
Fried	1.85[d]	302	1.69[d]	312	1.82[d]	296	1.83[c]	273

* Values were expressed as means of eight replicates. [a-d] Means within a column with no common superscripts differ at $P < 0.05$. Heritage brown leghorn, HB; Heritage white leghorn, HW; n-3 PUFA and lutein enriched, n-3/L.

In summary, we showed that the antioxidant activities as well as the contents of free amino acid and carotenoid of eggs were not affected under simulated retail storage conditions. The MDA content was also stable, indicating that no significant lipid oxidation occurred during storage. However, cooking significantly reduced the antioxidant activity and increased lipid oxidation, especially for stored eggs.

6.4. References

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CHAPTER 7 - Effect of Different Cooking Methods on Bioaccessibility and Digestive Stability of Egg Carotenoids as Studied in an *in vitro* Gastrointestinal System (TIM-1)[4]

[4]A version of this chapter has been submitted for publication. Nimalaratne, C., Savard, P., Gauthier, S.F., Schieber, A., & Wu, J. Effect of Different Cooking Methods on Bioaccessibility and Digestive Stability of Egg Carotenoids as Studied in an *in vitro* Gastrointestinal System (TIM-1). *Journal of Agricultual and Food Chemistry*.

CHAPTER 7

7.1. Introduction

Dietary carotenoids, especially lutein and zeaxanthin, have been reported to reduce the risk of age-related macular degeneration (AMD), which is a leading cause of irreversible vision loss in the elderly (Krinsky, Landrum, & Bone, 2003). In addition to various plant-derived dietary sources such as fruits, vegetables and green leafy vegetables, egg yolk serves as an important animal-derived source of lutein and zeaxanthin. Since carotenoids are lipophilic compounds, the digestible lipid matrix of the egg yolk makes it an ideal carrier to deliver highly bioavailable carotenoids; indeed, bioavailability of lutein from lutein enriched-egg yolk was found to be greater than from lutein supplements or spinach (Chung, Rasmussen, & Johnson, 2004; Handelman, Nightingale, Lichtenstein, Schaefer, & Blumberg, 1999). Through manipulation of hens' feed, egg yolk can be enriched with nutrients like carotenoids, vitamin E, selenium and omega-3 fatty acids (Surai, MacPherson, Speake, & Sparks, 2000).

To be bioavailable, a compound first needs to be released from its food matrix and micellarized into an absorbable (bioaccessible) form, which can then be taken up by the intestinal cells and metabolized (Faulks & Southon, 2005). Human studies would be the ideal approach to obtain most accurate information on nutrient bioavailability; however, high cost, technical difficulties and ethical constraints involved with human trials have increased the need of alternative methods (Faulks, Hart, & Brett, 2004; Hur, Lim, Decker, & McClements, 2011). As a result, *in vitro* digestion methodologies have been developed to mimic the *in vivo* human digestions and to assess the bioaccessibility of bioactive compounds which is usually followed by dialysis to determine the absorbable portion (Garrett, Failla, & Sarama, 1999; M. Minekus, Marteau, Havenaar, & Huis in 't Veld, 1995). Bioaccessibility varies among different types of carotenoids, whether a xanthophyll or a carotene, as well as among the different foods for a given carotenoid.

Several studies using *in vitro* digestion models (Chitchumroonchokchai, Schwartz, & Failla, 2004; Garrett *et al*., 1999; F Granado-Lorencio, 2007; O'Connell, Ryan, & O'Brien, 2007) as well as human studies (Hof & Brouwer, 1999; Schweiggert, 2014; Tyssandier *et al*., 2003) indicate that xanthophylls are more bioaccessible compared to carotenes. Furthermore, various food-related factors such as matrix, food composition, and cooking and processing conditions may change the micellarization efficiency (Moelants *et al*., 2012; Panozzo *et al*., 2013; Reboul *et al*., 2006; Stinco *et al*., 2012).

The TIM system is considered the most advanced *in vitro* digestive system developed to date, which can simulate the digestive processes of stomach and small intestines by TIM-1 and colon and microbial gut-derived flora by TIM-2 based on the studies performed with healthy individuals (M. Minekus *et al*., 1995). The main advantages of TIM-1 are its accuracy, reproducibility, and the possibility of collecting samples at any level of the gastrointestinal (GI) tract and at any time during the digestion. There are numerous studies using TIM-1 to assess bioaccessibility and digestive stability of carotenoids from various food products (Blanquet-Diot, Soufi, Rambeau, Rock, & Alric, 2009; Déat & Blanquet-Diot, 2009; Richelle & Sanchez, 2010; Van Loo-Bouwman *et al*., 2014).

However, the effect of cooking on digestive stability and bioaccessibility of egg carotenoids is poorly understood. Previously, we showed that cooking may affect the carotenoid profile of egg yolks (Nimalaratne, Lopes-Lutz, Schieber, & Wu, 2012). Therefore, the objectives of this study were to determine the effect of cooking and digestion on the stability and bioaccessibility of carotenoids and to characterize the carotenoid profile in digested eggs using TIM-1.

7.2. Material and methods

7.2.1. Chemicals

Pepsin (3200 U/mg, P6887, from porcine gastric mucosa), trypsin (from bovine pancreas, 7500 U/mg, T9201) and pancreatin (4xUSP, P1750, from porcine pancreas), lutein (xanthophyll from marigold) and zeaxanthin were obtained from Sigma-Aldrich (MO, USA). Lipase (150 units/mg (*Rhizopus oryzae* DF 15) was obtained from Amano Enzyme USA Co. The electrolyte solutions used for the digestion of proteins were gastric electrolyte solution (GES) containing NaCl (4.8 g/L), KCl (2.2 g/L), $CaCl_2$ (0.3 g/L), and $NaHCO_3$ (1.25 g/L) and small intestinal electrolyte solution (SIES) containing NaCl (5.0 g/L), KCl (0.6 g/L), and $CaCl_2$ (0.3 g/L). Fresh pig bile was collected from Olymel slaughterhouse (Vallée-Jonction, QC, Canada), aliquoted for individual TIM experiments and stored at -20 °C until use. Bile bladder had a size of minimal 8 cm in length. HPLC grade solvents (methanol, ethyl acetate, petroleum ether and *tert*-butyl methyl ether) were purchased from Fisher Scientific (Ottawa, ON, Canada).

7.2.2. Sample Preparation

Fresh eggs (n=180) were obtained from the Poultry Research Centre of University of Alberta, (Edmonton, Canada). Eggs were divided into three groups of 60 each and subjected to boiling, frying and scrambling. Boiled and fried eggs were prepared using the same cooking conditions that were reported previously (Nimalaratne *et al*., 2012) and the yolks were separated from egg white. To prepare scrambled eggs, yolks were separated and combined and stirred on a pre-heated (205 °C) model SK200TY non-stick frying pan (Black & Decker Canada Inc., Brockville, ON, Canada) by stirring yolks for 1 min. Each sample of cooked egg yolks was divided into two subgroups. Samples were vacuum packed, covered with aluminium foil to protect carotenoids

from light exposure, and frozen immediately at -20 °C. On the next day, all samples were shipped in a Styrofoam box along with dry ice to the Institute of Nutrition and Functional Foods (INAF) at Laval University (Quebec City, QC, Canada) where they were stored at -80 °C until subjected to digestion.

7.2.3. TIM-1 Dynamic Gastrointestinal Model

TNO's gastrointestinal model-1 (TIM-1), which has been previously described in detail (M. Minekus *et al*., 1995) and commercialized by TNO Nutrition and Food Research (Zeist, Netherlands), was used for this study. In brief, the computer-controlled system consists of four compartments made of a glass outer wall and a flexible inner wall to represent stomach, duodenum, jejunum and ileum. These compartments are connected by peristaltic valve-pumps, which facilitate the passage of a constant volume of chyme during each open and close cycle. The space between the two walls is filled with water at 37 °C to mimic body temperature. Changing the water pressure enables peristaltic movements and churning of chyme by alternated compression and relaxation of the inner walls. Previously formulated solutions to mimic salivary, gastric, biliary, and pancreatic secretions are introduced into the system via a computer-controlled pumping system. Two hollow-filter membranes are connected to jejunum and ileum to simulate the absorption of nutrients/drug and water from chyme. The volume in each compartment and pH, which is regulated using hydrochloric acid and/or sodium bicarbonate, are continuously monitored and controlled.

7.2.4. Digestion of Samples with TIM-1

Approximately 200 g of cooked egg sample was homogenized with 200 g of distilled water to prepare 'meal' using a hand-held blender model CSB-79C (Cuisinart, Woodbridg, ON, Canadae)

for 12 sec. A sample of 100 g from the meal was taken out as a control and the rest (300 g) was immediately fed into the gastric compartment of the TIM-1 system. The digestion was carried out using predetermined parameters of TIM-1 modified from Speranza *et al*., (2013) and adapted to reproduce the digestion of a semi-solid meal in a healthy human adult (Table 7.1). The jejunal and ileal compartments were connected to specific filter membranes (MiniKros module M80S-300-01P, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) that separate the micellar phase from the fat phase in order to determine bioaccessibility of lipophilic products (Mans Minekus *et al*., 2005; Reis *et al*., 2008). The mixed micelles that contain the bioaccessible lipophilic fraction are able to pass through these membranes, while the undigested fat is retained. These bioaccessible fractions are collected in separate pouches kept on ice. After the passage of the chyme throughout the system, the residual digestion mixture is discharged as the effluent, which was kept on ice during the experiment. The digestion was carried out under subdued light to prevent photo-degradation of carotenoids. All solutions/secretions used during the digestion were purged with nitrogen, and continuous nitrogen flow to the jejunal, ileal and effluent compartments was maintained to minimize carotenoid oxidation and to reproduce the *in vivo* luminal conditions. Duplicated digestions were carried out for each cooked egg sample. A blank digestion was also performed using the same parameters with 200 g of distilled water in place of egg sample in order to estimate the carotenoids from digestive secretions and bile found in each sample.

7.2.5. Sampling Location and Time

During the total digestion time of 300 min, samples were obtained from different compartments as follows: 8 g of stomach chyme at 120 min and 8 g samples from duodenal, jejunal and ileal chyme at 150 min were taken out. Jejunal filtrate, ileal filtrate and effluent were collected from

0-150 min and from 150-300 min into separate pouches kept on ice. The residual chyme samples in duodenal, jejunal and ileal compartments at the end of the digestion were also collected. Samples were weighed, aliquoted and immediately transferred to a -80 °C freezer in airtight containers. All samples were freeze-dried and transported to the University of Alberta (AB, Canada) with ice packs, where they were stored at -20 °C until analysis.

7.2.6. Analysis of Carotenoids in Digested Samples

Carotenoids in each sample were extracted as described previously with modifications (Schlatterer & Breithaupt, 2006). Briefly, 500 mg of sample was accurately weighed in to a micro centrifuge tube and extracted twice with 0.5 mL of a ternary solvent mixture consisting of methanol: ethyl acetate: petroleum ether (1:1:1 v/v/v) containing 0.1% BHT. The supernatants were combined in micro centrifuge tubes, evaporated under nitrogen and reconstituted in 0.5 mL of acetone. The acetone extracts were kept at -80 °C for ~ 1 hr followed by centrifugation at 16,500 *g* for 30 sec and the supernatants were immediately transferred to a separate micro centrifuge tube, evaporated under nitrogen and reconstituted in 200 µL of methanol. The extracts were filtered through a 0.45 µm nylon syringe filter and analysed by HPLC using a Waters 600 HPLC system (Waters, Millford, MA, USA) equipped with a 2702 thermoautosampler, a binary gradient pump and a 2998 photodiode array detector. The separation of carotenoids was performed on a C_{30} reversed-phase column (YMC 250 mm × 4.6 mm, i.d., 5 µm) using the conditions reported before (Nimalaratne *et al*., 2012). Duplicate analyses were performed. To protect carotenoids from light and oxygen induced degradation, dim light conditions were used during sample handling, extraction and analysis, and solvents used were purged with nitrogen. Carotenoids were identified based on their UV spectra, retention time and order of elution and quantified using seven point standard calibration curves.

Table 7.1. Parameters of the TIM-1 system when simulating digestive conditions of a healthy adult after intake of a high fat semi-solid meal ("fed state").

Compartment	Starting content/Secretions/Filtration fluid	Volume (mL)	pH	$t_{1/2}$ / β
Stomach	Starting content: • 10 g of gastric juice (pH 2) 10400 U of pepsin and 400 U of lipase in GES Secretions: • 1040 U/mL of pepsin and 40 U/mL of lipase in GES circulating at 0.5 mL/min • Water or HCl (1 M) circulating at 0.5 mL/min	310	0/5.5 10/5.0 20/4.2 40/2.8 60/2.1 90/1.8 120/1.7 300/1.7	70 / 2
Duodenum	Starting content: • 15 g of a pancreatin solution (21 %), 30 g of fresh bile, 1 mL of trypsin solution (2 mg/mL) and 15 g of SIES (pH 7) Secretions: • pancreatin solution (21 %) circulating at 0.25 mL/min • fresh bile circulating at 0.50 mL/min • SIES or $NaHCO_3$ (1 M) circulating at 0.25 mL/min • SIES circulating at 3.2 mL/min	60	6.3	160 / 1.6
Jejunum	Starting content: • 40 g of SIES, 80 g of fresh bile and 40 g of pancreatin solution (21 %) Secretions: • $NaHCO_3$ (1 M) if necessary	160	6.5	

	• SIES containing 10 % of fresh bile circulating at 3.2 mL/min Filtration fluid: • SIES circulating at 4.5 mL/min		
Ileum	Starting content: • 160 g of SIES Secretions: • $NaHCO_3$ (1 M) if necessary • SIES circulating at 3.0 mL/min Filtration fluid: • SIES circulating at 4.5 mL/min	160	7.4

Gastric and ileal deliveries are modeled with a power exponential formula: $f = 1–2^{-(t/t1/2)\beta}$, where f represents the fraction of meal delivered; t, the time of delivery; $t^{1/2}$, the half-time of delivery; and β, the coefficient describing the shape of the curve. GES (Gastric electrolyte solution), SIES (Small intestine electrolyte solution)

7.2.7. Calculations and Statistical Analysis

The recovery of carotenoid compounds (separately for lutein and zeaxanthin) in each cooked egg sample was calculated as a percentage of the total amount of carotenoid compounds fed into the system using following equation.

$$Recovery\ of\ carotenoids\ (\%) = \frac{total\ amount\ in\ jejunal\ filtrate + ileal\ filtrate + effluent + chyme\ residue\ at\ 300\ min}{(total\ amount\ fed\ into\ the\ system - the\ amount\ detected\ during\ blank\ digestion} x\ 100$$

All data were analyzed by Analysis of Variance (ANOVA) followed by Tukey's multiple range test using Statistical Analysis System Software (SAS version 9.3, SAS Institute Inc., Cary, NC, USA). Significance of differences was defined at the 5% level ($p < 0.05$).

7.3. Results and discussion

7.3.1. Carotenoid Profile of Eggs

The main carotenoid isomers found in undigested egg yolk were all-*E* lutein, all-*E* zeaxanthin, 13'-*Z* lutein and 13-*Z* zeaxanthin. The other carotenoids usually found in eggs such as all-*E* canthaxanthin and all-*E*-β-apo-8' carotenoic acid ethyl ester were only found in trace amounts (Figure 7.1). The eggs used in this study did not use synthetic carotenoids as poultry feed additives. This might explain their absence in eggs as the egg carotenoid profile is highly dependent on hens' diet (Schlatterer & Breithaupt, 2006). The qualitative HPLC chromatographic profile of the egg carotenoid isomers remained unchanged during the digestion as no new isomer formation was observed (Figure 7.1). 13'-*Z* lutein and 13-*Z* zeaxanthin were found in most of the samples, but were below the limit of quantitation in the duodenal, jejunal and ileal chyme. Thus, the total recovery of these *cis* isomers could not be calculated. Coinciding with previous reports (Fernando Granado-Lorencio *et al*., 2007, 2010), no significant *trans*-*cis* isomerization of carotenoids was observed during the digestion due to the high recovery of *trans* lutein and zeaxanthin as well as the total quantity of *cis* isomers in filtrates and effluent were much lower than the amounts in the meal. Therefore, in this study we mainly focused on all-*E* lutein and all-*E* zeaxanthin levels in the gut during the digestion; unless mentioned otherwise, lutein and zeaxanthin represent the *trans* form of the respective carotenoid.

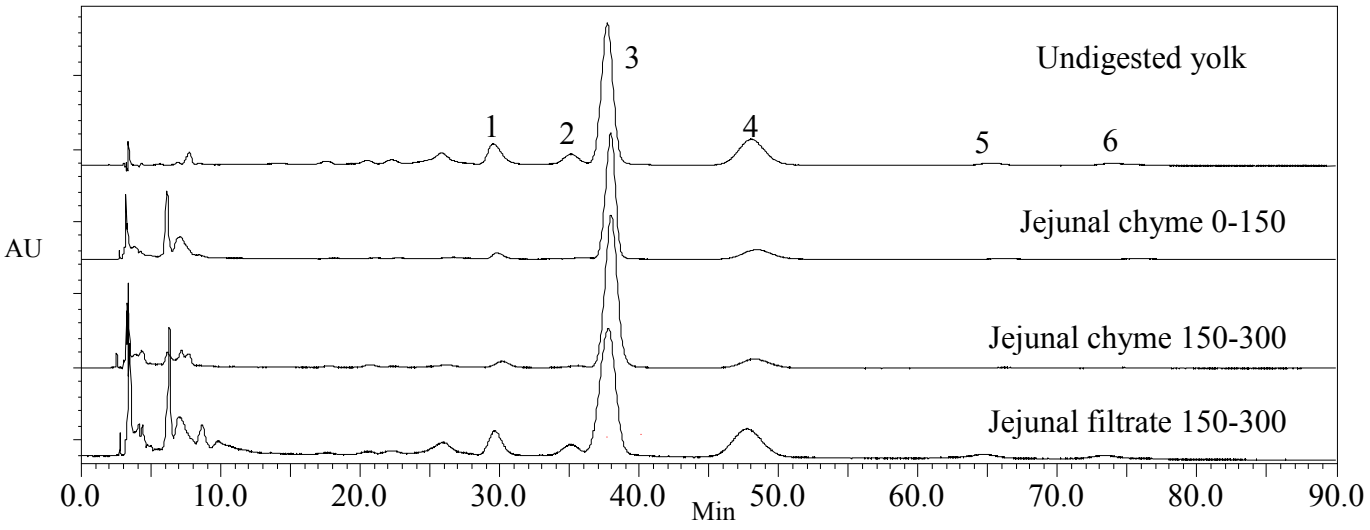

Figure 7.1. Representative HPLC chromatograms of egg yolk carotenoids from different intestinal compartments during digestion. Peak identification (1) 13'-*Z* lutein, (2) 13-*Z* zeaxanthin, (3) All-*E* lutein, (4) All-*E* zeaxanthin, (5) All-*E* canthaxanthin, (6) All-*E* β-apo-8'-carotenoic acid ethyl ester

7.3.2. Digestive Stability of Carotenoids and Distribution in TIM Compartments during Digestion

Only trace amounts of endogenous lutein and zeaxanthin from bile and/or other intestinal secretions were observed in the samples from the blank digestion, which were below the quantitative limits of HPLC. Hence, the distribution and recovery of carotenoids were calculated as a percentage of carotenoid content present in eggs fed into the TIM-1. Overall, both lutein and zeaxanthin were stable during digestion, with average recoveries of 90% and 88% observed for all-*E* lutein and all-*E* zeaxanthin, respectively. At half time of the digestion (150 min), approximately 37% of lutein irrespective to the type of the sample exited the small intestine

(indicated as 'effluent') without absorption (Figure 7.2A). Around 16% and 8% of lutein were passed through the hollow fibre membrane as bioaccessible lutein into the jejunal and ileal filtrates respectively. Most of the remaining lutein was still in the jejunal and ileal compartments, while only about 2% were present in the duodenum. No lutein was detected in the stomach chyme samples even at 120 min through digestion and by 150 min the stomach is completely free from meal. All-*E* zeaxanthin had distribution profiles similar to those of lutein, however, a comparatively higher proportion (~50%) in scrambled eggs was found in effluent as non-bioaccessible zeaxanthin. The average recovery of zeaxanthin from fried eggs at 150 min was 69.7%, much lower than those of boiled and scrambled samples. This is because we failed to quantify the zeaxanthin contents in duodenal, jejunal and ileal chyme samples from one of the two fried egg digestions as they were below the quantifiable limits of HPLC. Consequently, the calculated recovery at 150 min was lower than the true recovery of zeaxanthin. At the end of the digestion (at 300 min), almost 50% of the total lutein was found in effluent followed by 30% in jejunal filtrates. Around 10% of lutein was in ileal filtrate while only a small amount remained in duodenal, jejunal and ileal compartments.

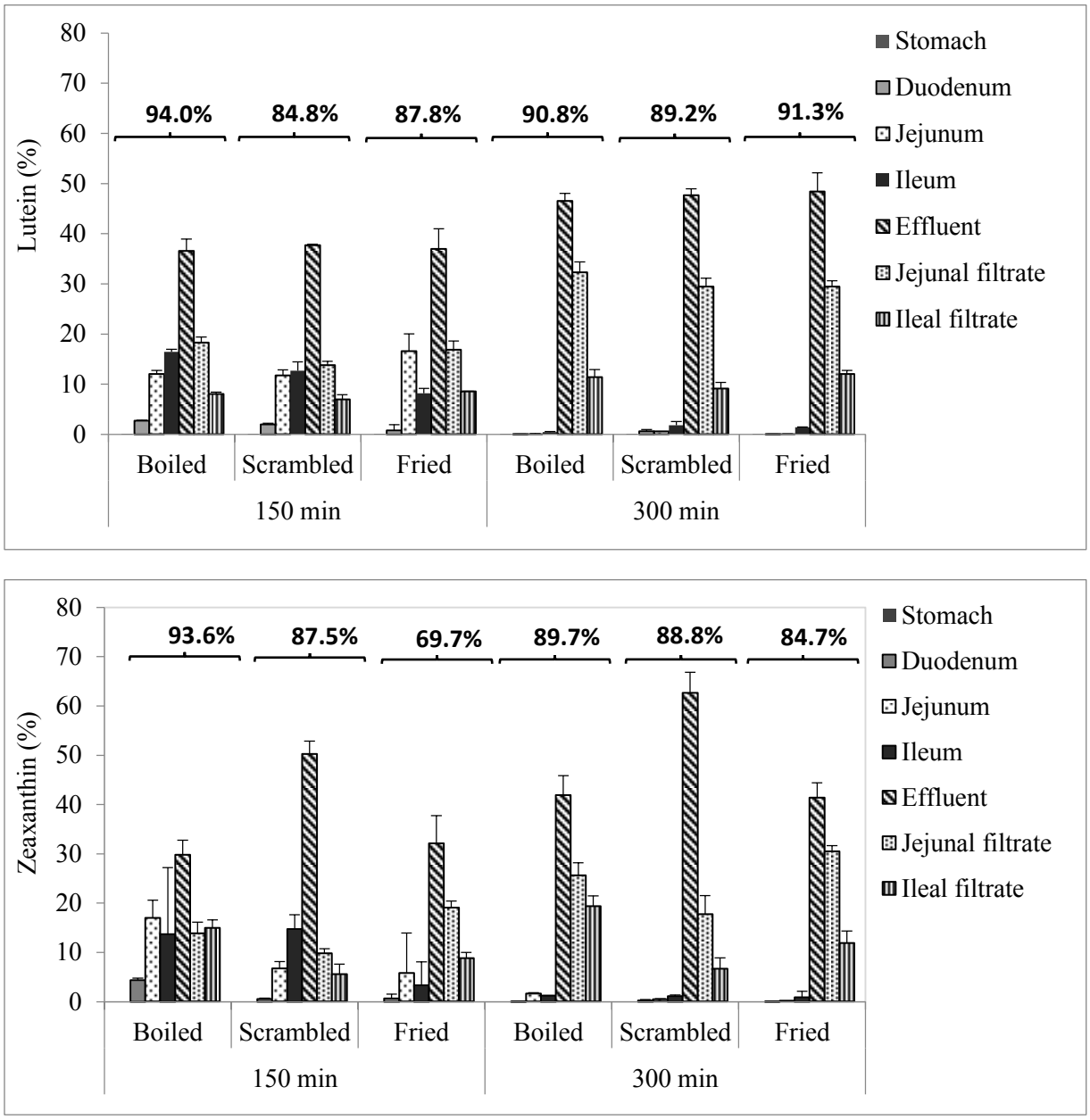

Figure 7.2. Total recovery (indicated on top of the bars) and distribution of all-*E* lutein (A) and all-*E* zeaxanthin (B) in the gut during digestion (as a % of the feeding) (average of two runs)

A similar distribution pattern was observed with zeaxanthin except that a higher amount (~63%) was found in the effluent in scrambled eggs (Figure 7.2B), apparently the reason for comparatively lower bioaccessibility (Figure 7.3), which will be discussed below.

Even though no reports are available regarding the gut distribution and/or stability of lutein and zeaxanthin from egg yolk, these carotenoids have been reported as highly stable during gastrointestinal digestion regardless of the food source. Blanquet-Diot *et al*., (2009) used TIM-1 to compare the digestive stability of xanthophylls and carotenes and found that lutein and zeaxanthin were stable throughout the digestion and the digestive stability of xanthophylls is higher than that of carotenes. Studies which used other *in vitro* digestion methods also showed a higher digestive stability (>80%) of lutein and zeaxanthin derived from different food sources (Chitchumroonchokchai *et al*., 2004; Garrett *et al*., 1999; Fernando Granado-Lorencio *et al*., 2007; Reboul *et al*., 2006).

7.3.3. Bioaccessibility of Carotenoids from Cooked Eggs

Bioaccessibility of carotenoids was the total amount of ileal and jejunal filtrates expressed as a percentage of the carotenoid in starting meal. According to Etcheverry, Grusak, & Fleige (2012), there are principally four *in vitro* methods for measuring bioaccessibility and/or bioavailability: solubility, dialyzability, or a gastrointestinal model (e.g.,TIM) for bioaccessibility, and the cells culture for bioavailability. There exist no *in vitro* digestion models that have the capability to mimic perfectly the complex environment of the gastrointestinal (GI) tract. However, TIM-1 is the only *in vitro* digestion model with the capacity to simulate the dynamic conditions of the stomach and small intestine during digestion (Kostewicz *et al*., 2014). As shown in Figure 7.1, the isomeric profiles of carotenoids were similar in both non-bioaccessible (jejunal chyme 0-150 min, 150-300 min) and bioaccessible samples (jejunal filtrate 150-300 min), indicating both

trans and *cis* isomers of carotenoids passed through the hollow fibre membranes into the bioaccessible filtrates. In boiled, fried and scrambled eggs, the bioaccessibility of lutein was 44%, 41% and 39% while that of zeaxanthin was 45%, 42% and 24% respectively (Figure 7.3).

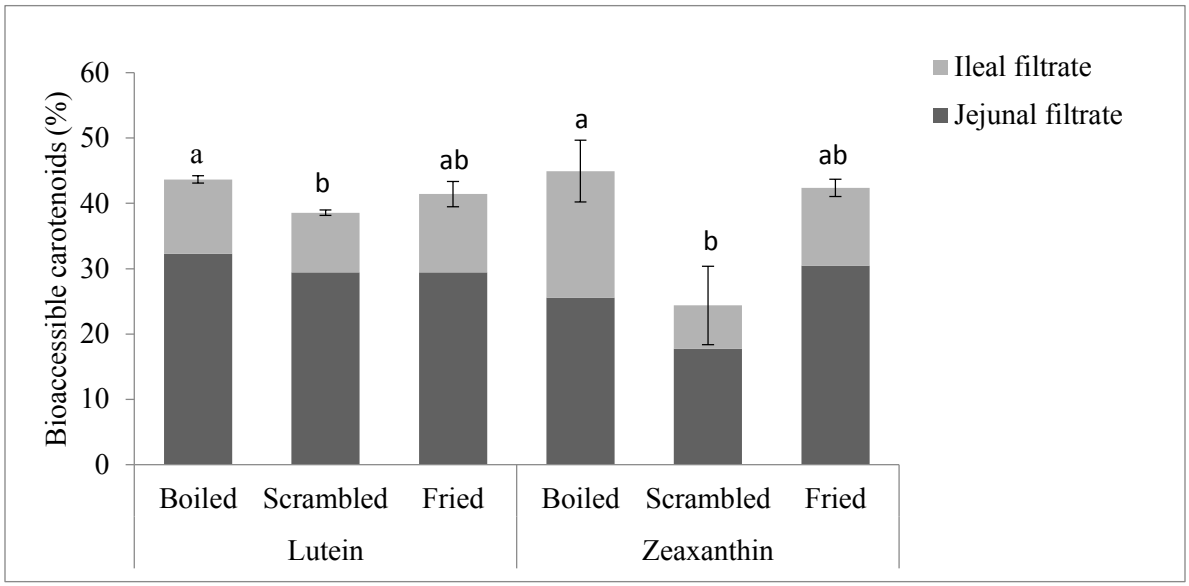

Figure 7.3. Cumulative carotenoids in jejunal and ileal filtrates (bioaccessible carotenoids) as a percentage of the feeding at the end of the digestion (n=2; [a-b] different letters denote significant difference (p <0.05))

Total bioaccessible carotenoids were higher in the jejunal filtrate than in the ileal filtrate. A similar trend was observed in the recent studies who used TIM-1 to study the effect of fat on blueberry anthocyanins (Ribnicky *et al*., 2014) and β-carotene bioaccessibility (Van Loo-Bouwman *et al*., 2014). Although we did not find any study on lutein bioaccessibility using TIM-1, there are many other reports on lutein bioaccessibility using other *in vitro* digestion models. Garret *et al* developed an *in vitro* digestion model and found the lutein bioavailability (solubility assay+cells culture) in a standard baby food to be 25-40% (Garrett *et al*., 1999) which is

consistent with our results. Using the same model, O'Connell demonstrated that lutein from fruits including orange, kiwi, grapefruit and honeydew melon (100-109%) is more bioaccessible compared to dark green vegetables, spinach (19%) and broccoli (38%) (O'Connell *et al*., 2007). However, Chitchumroonchokchai *et al*., (2004) found a much higher lutein micellarization (53%) in microwave cooked spinach using solubility assay coupling with Caco-2 cells, presumably due to the disruption of cell walls. Bioaccessibility of lutein from pure durum wheat pasta amounted to 71%, whereas from egg pasta it was 57% (Werner & Böhm, 2011), which seems contradictory to the fact of highly bioavailability of egg lutein. It was speculated that egg lutein was incorporated into the complex mixture of proteins and starch during pasta processing, leading to low bioaccessibility. Lutein fortified whole, semi-skimmed and skimmed milk showed bioaccessible levels of 46.5%, 45.8% and 19.7%, whereas in whole, semi-skimmed and skimmed yogurts, it was 47.5%, 38.3% and 17.8% respectively (Xavier, Mercadante, Garrido-Fernández, & Pérez-Gálvez, 2014), and this data is comparable to bioaccessibility levels observed in this study.

The bioaccessibility of both lutein and zeaxanthin was affected by the type of cooking (Figure 7.3). Lutein and zeaxanthin from scrambled eggs yielded significantly lower bioaccessibility compared to boiled eggs, but were not different from fried eggs. The exact reason for this difference is not clear, but presumably the chemical and structural changes of proteins and lipoproteins in egg yolk occurred during different cooking conditions may have influenced the micellarization efficiency, that is, the transfer of carotenoids to the filtrates. Textural differences between the cooked eggs can be a considerable factor on micellarization as boiled eggs are easily blended with water compared to fried and scrambled egg. Particle size, food processing and food preparation (Moelants *et al*., 2012; Panozzo *et al*., 2013; Reboul *et al*., 2006; Stinco *et al*., 2012)

have been reported as influencing factors on carotenoid bioaccessibility. Panozzo *et al*. (2013) showed bioaccessibility of carotenoids from tomato pulp was affected by homogenization in two main ways; a) decreased particle size which enables release of carotenoids; and b) increased consistency due to formation of fibre network which entrap the carotenoids. Similarly, the industrial extracted orange juice resulted in smaller particle sizes increased the relative bioaccessibility compared to hand extraction and accordingly (Stinco *et al*., 2012). The composition and degree of saturation of fatty acids is also considered an important factor on carotenoid bioaccessibility and bioavailability (Gleize *et al*., 2013; Goltz, Campbell, Chitchumroonchokchai, Failla, & Ferruzzi, 2012; Nidhi & Baskaran, 2010). Gleize *et al*., 2013 observed that saturated and mono-unsaturated short chain fatty acids (MUFA) lead to formation of small micelles and higher bioaccessibility of lutein and zeaxanthin compared to the long-chain polyunsaturated fatty acids (PUFA). The significant inverse relationship between the micelle size and bioaccessibility was attributed to increased specific surface area with smaller micelle. They hypothesized that due to the polar nature, xanthophylls located at micelle surface, and increased surface area increases amount of xanthophylls that can be incorporate into the micelles. Different cooking methods resulted in diverse fatty acids profiles in egg yolk (Cortinas, Galobart, Barroeta, Baucells, & Grashorn, 2003; USDA, 2014). The amount of PUFA in scrambled eggs is significantly higher compared to boiled eggs, which may lead to formation of larger micelles and lower bioaccessibility. This can also explain our observations on bioaccessibility of carotenoid from eggs after different cooking methods.

TIM-1 is so far considered the most advanced digestive model simulating the *in vivo* conditions of human gut and very useful in predicting the fate of nutrients, pharmaceuticals etc (Guerra *et al*., 2012). However, the data obtained through *in vitro* digestion models on the bioaccessibility

of carotenoids may not directly be applied as bioavailability in humans and should be interpreted with caution. Based on previously published studies, Reboul *et al*. (2006) made a comparison between the bioaccessibilities obtained using *in vitro* digestive models and bioavailability values measured in healthy humans. They found that there is a significant relationship ($r = 0.98$, $p < 0.0001$) between bioaccessibility ratios measured *in vitro* and the mean bioavailability ratios. In contrast, Granado *et al*. (2006) showed that the bioaccessibility of carotenoids and vitamin E from broccoli under *in vitro* conditions does not fully explain the changes they observed in healthy. Another report stated that, although the micellarization efficiency of lutein from human milk and infant formula was similar, accumulation efficiency of human milk lutein was 4.5 times higher by Caco-2 human intestinal cells (Lipkie *et al*., 2014).

In summary, we studied the effect of different domestic cooking methods on the stability and bioaccessibility of carotenoids from egg yolk. In alignment with the previous findings, lutein and zeaxanthin were stable during the *in vitro* digestion. Digestive stability was not affected by the different types of cooking, whereas scrambling resulted in significantly lower bioaccessibility compared to boiled eggs in both lutein and zeaxanthin. To the best of our knowledge, this is the first study to investigate the bioaccessibility of egg yolk carotenoids affected by the cooking methods. The results presented here provide detailed information on digestive stability and bioaccessibility of egg carotenoids under various thermal processing conditions and may be useful to estimate the bioavailable carotenoids from eggs and egg-containing foods.

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CHAPTER 8 - Antioxidant Activity of Egg Yolk Increased After Simulated Gastrointestinal Digestion Using the TNO Gastrointestinal Model (TIM-1)

CHAPTER 8

8.1. Introduction

Oxidative stress is associated with initiation and propagation of many chronic diseases for example atherosclerosis, cancer, diabetes, aging, Alzheimer's disease and other degenerative diseases in humans (Finkel & Holbrook, 2000) . An imbalance between free radical formation and antioxidant scavenging leads to oxidative stress (Valko *et al*., 2007). Intake of antioxidants via foods or supplements is believed to be effective in reducing oxidative stress. Food derived natural antioxidants have attracted great attention owing to their safety over synthetic antioxidants. Phenolic compounds, flavonoids, plant or animal origin peptides are some of the food-derived antioxidants which have been widely investigated (Duthie & Crozier, 2000; Samaranayaka & Li-Chan, 2011). Among the foods of animal origin, eggs are considered to be a highly nutritious food commodity rich in bioactive compounds including antioxidants (Huopalahti, López-Fandiño, Anton, & Schade, 2007). In recent years, antioxidant activity of various egg-derived compounds such as peptides, proteins, phospholipids, carotenoids and vitamins has been reported (Dávalos, Miguel, Bartolomé, & López-Fandiño, 2004; Sarmadi & Ismail, 2010; Xu, Katayama, & Mine, 2007).

Both food processing and gastrointestinal digestion can affect the activity of a certain antioxidant compound (Dewanto, Wu, & Liu, 2002; You, Zhao, Regenstein, & Ren, 2010). Exposure to heat, light, and oxygen during light would enhance or reduce the activity of antioxidants (Jiménez-Monreal, García-Diz, Martínez-Tomé, Mariscal, & Murcia, 2009; Turkmen, Sari, & Velioglu, 2005). Human gut is known as an oxidation site which generates new reactive oxygen species (ROS), during the digestion process (Halliwell, Zhao, & Whiteman, 2000). Because of the extreme pH conditions and exposure to food-derived metal ions such as iron, copper, hydrogen peroxide, haem, lipid peroxides, and nitric oxide, gastrointestinal digestion could affect the

activity of antioxidant compounds (Alexandropoulou, Komaitis, & Kapsokefalou, 2006; Sannaveerappa, Westlund, Sandberg, & Undeland, 2007; Srigiridhar, Nair, Subramanian, & Singotamu, 2001). Since human studies are costly and associated with technical difficulties and ethical constraints, *in vitro* digestion models simulating the human digestive tract are being extensively used in recent times. Among many gastrointestinal models that have been used to simulate the digestion process, TNO's intestinal model (TIM-1) is considered the most advanced *in vitro* digestive system developed to date. It has been previously used to assess the bioaccessibility of nutrients/drugs (Blanquet *et al*., 2004; Nabil, Gauthier, Drouin, Poubelle, & Pouliot, 2011; Öhrvik, Öhrvik, Tallkvist, & Witthöft, 2010; Ribnicky *et al*., 2014) and activity of bioactive compounds (Krul *et al*., 2001; Mateo Anson, Havenaar, Bast, & Haenen, 2010) as affected by the digestion.

Our previous study revealed that free amino acids (FAA), especially aromatic amino acids contribute to the antioxidant properties of egg yolk and domestic cooking methods such as boiling and frying reduce the antioxidant activity (Nimalaratne, Lopes-Lutz, Schieber, & Wu, 2011). However, the effect of gastrointestinal digestion on the antioxidant activity is unclear. On the one hand, during the digestion breakdown of proteins may generate FAA, which might leads to increased antioxidant activity and on the other hand the extreme pH conditions and digestive enzymes may cause degradation of some amino acids and other antioxidant compounds such as carotenoids and vitamin E. Therefore, this study was designed to determine the content of bioaccessible FAA and changes of antioxidant activity of cooked eggs after gastrointestinal digestion.

8.2. Material and Methods

8.2.1. Chemicals

HPLC-grade methanol, monobasic phosphate and hydrochloric acid were purchased from Fisher Scientific (Ottawa, ON, Canada). Pepsin from porcine gastric mucosa (3200 U/mg, P6887), trypsin from bovine pancreas (7500 U/mg, T9201) and pancreatin from porcine pancreas (4xUSP, P1750), 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH) and trichloroacetic acid were obtained from Sigma-Aldrich, (MO, USA). Lipase (150 units/mg (Rhizopus oryzae DF 15) was obtained from Amano Enzyme USA Co. Fluorescein disodium and Trolox were obtained from Acros Organics (Morris Plains, NJ). The electrolyte solutions used for the digestion of proteins were gastric electrolyte solution (GES) containing NaCl (4.8 g/L), KCl (2.2 g/L), $CaCl_2$ (0.3 g/L), and $NaHCO_3$ (1.25 g/L) and small intestinal electrolyte solution (SIES) containing NaCl (5.0 g/L), KCl (0.6 g/L), and $CaCl_2$ (0.3 g/L). Fresh pig bile was collected from Olymel slaughterhouse (Vallée-Jonction, QC, Canada), aliquoted for individual TIM experiments and stored at -20 °C until use.

8.2.2. Cooking of Eggs

Fresh, non-fertilized chicken eggs (n=240) were obtained from the Poultry Research Centre farm of the University of Alberta (Edmonton, AB, Canada). Eggs were divided into four sets of 60 each. Three sets of eggs were subjected to boiling, frying and scrambling. Boiled and fried eggs were prepared as described previously (Nimalaratne *et al*., 2011) and the yolks were separated from egg white. To prepare scrambled eggs, yolks were separated and combined and stirred on a pre-heated (205 °C) non-stick frying pan (model SK200TY, Black & Decker Canada Inc., Brockville, ON, Canada) for 1 min. The remaining set of eggs also subjected to boiling using the

same conditions as above and used as the whole eggs. Each sample was divided into two subgroups, vacuum packed, covered with aluminium foil and placed immediately at -20 °C. On the next day, all samples were shipped with dry ice to the Institute of Nutrition and Functional Foods (INAF) at Laval University (Quebec City, QC, Canada) where they were stored at -80 °C until the digestion.

8.2.3. Preparation of Samples for Digestion in TIM-1

Frozen samples were thawed by placing in the refrigerator the day before the digestion. Approximately 200 g of cooked egg sample was homogenized with 200 g of distilled water to prepare 'meal' using a hand-held blender model CSB-79C (Cuisinart, Woodbridg, ON, Canada) for 12 sec. A sample of 100 g from the meal was taken out as a control and the rest was immediately fed into the gastric compartment of the TIM-1 system.

8.2.4. Digestion of Eggs using TIM-1

In here, we used TIM-1 (TNO Nutrition and Food Research, Zeist, Netherlands) which was described before (Minekus, Marteau, Havenaar, & Huis in 't Veld, 1995). In brief, TIM-1 consists of four compartments to represent stomach, duodenum, jejunum and ileum composed of a flexible inner silicone jacket surrounded by a glass capsule and connected with each other by peristaltic valve-pumps. The jejunal and ileal compartments are also connected to hollow-fiber membranes to simulate the absorption of bioaccessible digestion products and water from chyme. Gastric, biliary, and pancreatic secretions are previously formulated and introduced into the system via computer-controlled pumping system. The volume in each compartment and pH, which is regulated using hydrochloric acid and/or sodium bicarbonate, are continuously monitored and controlled.

The digestion was carried out using predetermined parameters adapted and modified from Speranza *et al*. (Speranza *et al*., 2013) to reproduce the digestion of a semi-solid meal in a healthy human adult (see Table 7.1). The jejunal and ileal compartments were connected to two semi-permeable hollow fibre membrane units (MiniKros module M80S-300-01P, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) to mimic the absorption of digested compounds in chyme, which are referred to as the bioaccessible compounds.

All solutions/secretions used during the digestion were purged with nitrogen, and a continuous nitrogen flow to the intestinal compartments was maintained to reproduce the *in vivo* luminal conditions. Gastrointestinal *in vitro* digestions were performed in duplicate. A blank digestion was also performed using the same parameters with 200 g of distilled water in place of egg sample.

8.2.5. Sampling Location and Time.

A complete digestion lasted for 300 min and the samples were obtained from different compartments as follows: 8 g of stomach chyme at 120 min and 8 g samples each from duodenal, jejunal and ileal chyme at 150 min. Dialysate samples from the jejunal and ileal compartments were collected from 0-150 min and from 150-300 min into separate pouches kept on ice during the digestion. The residual chyme samples in duodenal, jejunal and ileal compartments at the end of the digestion were also collected (Figure 8.1). Samples were weighed, aliquoted and immediately transferred to a -80 °C freezer in airtight containers. All samples were freeze-dried and transported to the University of Alberta (Edmonton, AB, Canada) on dry ice. Freeze dried samples were stored at -20 °C until further analysis.

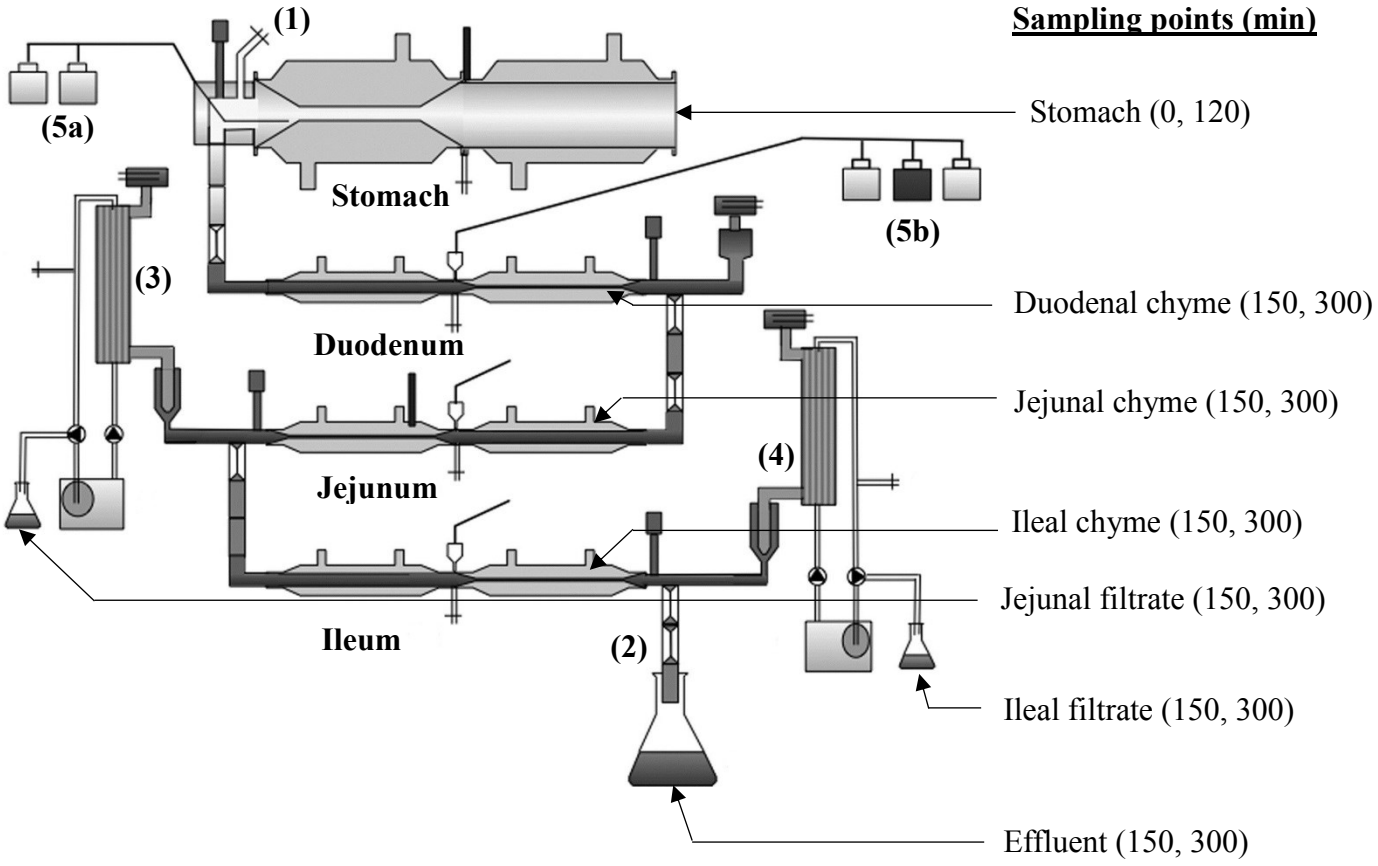

Figure 8.1. Schematic diagram of the *in vitro* gastrointestinal model, TIM-1 and sampling points (1) food inlet, (2) ileal colorectal valve, (3) hollow fiber membrane from jejunum, (4) hollow fiber membrane from ileum, (5a) and (5b) secretion pumps. Modified and reprinted with permission from ref (Speranza *et al*., 2013). Copyright 2013 American Chemical Society

8.2.6. Oxygen Radical Absorbance Capacity-Fluorescein (ORAC-FL) Assay

Freeze dried digested samples were weight (50 mg) into a microcentrifuge tube and 0.5 mL of acidified (pH 1.5, HCl) 80% methanol was added. Samples were extracted with vortex mixing followed by centrifugation at 6000 *g* for 10 min (Centrifuge 5418, Eppendorf, Hamburg, Germany). The supernatants were evaporated and reconstituted with 200 μL of methanol. The ORAC-FL assay was performed according to the method of Ou *et al*. (Ou, Hampsch-Woodill, & Prior, 2001) modified by Dávalos *et al*. (Dávalos *et al*., 2004). The reaction was carried out in 96F black, untreated 96-well microplates (Nunc, Roskilde, Denmark) and Trolox (2-16 μM) was used as standard. For each experiment, 80 mM AAPH and 200 nM fluorescein in 75 mM phosphate buffer at pH 7.4 were prepared. For each assay, 20 μL of sample and 80 μL of phosphate buffer (or 100 μL of Trolox standard solutions at final concentrations of 1−8 μM) were added to the well followed by the addition of 50 μL of the fluorescein solution. Final assay mixture was incubated in a Fluoroskan Ascent microplate reader (Thermo Electron Corp., Vantaa, Finland) at 37 °C for 10 min and 50 μL of APPH solution (80 mM) were added to each well using an automated dispenser. After mixing for 5 s, the fluorescence was recorded at 1 min intervals for 100 min at excitation and emission wavelengths of 485 and 538 nm, respectively. The area under curve (AUC) was calculated for each sample by using Graph pad Prism software (trial version). Regression equations obtained from the Trolox standard curve was used to calculate the ORAC-FL value for each assay. All assays were conducted in triplicates. Final ORAC-FL values were expressed as micromoles of Trolox equivalent (TE) per mg of yolk (μmol TE/mg).

8.2.7. Determination of FAA in Egg Yolk

Approximately 150 mg of the freeze-dried egg yolk was extracted with 1 mL of 6% trichloroacetic acid by vortex mixing for 2 min (Ohkubo *et al*., 2006). The mixture was centrifuged at 6000 *g* for 20 min (Centrifuge 5418, Eppendorf, Hamburg, Germany) and the supernatant was filtered with 0.22 μm Nylon syringe filter (Mandel Scientific Corp., Guelph, ON, Canada). FAA profile was carried out according to the method described by Sedgwick *et al*. (Sedgwick *et al*., 1991) using *o*-phthaldialdehyde (OPA) as the derivatizing agent. The method used to determine FAAs in here does not detect proline and cysteine because of the derivatizing agent (*o*-phthaldialdehyde) used is not reacting with proline and yields a weaker fluorescence signal with cysteine (Cooper & Turnell, 1982). Total FAA content of the samples was adjusted by subtracting the total FAA content of the blank sample. Duplicate analyses were performed.

8.2.8. Statistical Analysis

To determine the differences of antioxidant activity and FAA contents in different samples, the data were subjected to analysis of variance (ANOVA) followed by Tukey's multiple range test using Statistical Analysis System Software (SAS version 9.3, SAS Institute, Cary, NC, USA). Significance of differences was defined at 5% level ($p < 0.05$)

8.3. Results and Discussion

8.3.1. Changes of FAA Content during the Digestion.

The FAA contents of all egg samples increased after digestion (Figure 8.2). Undigested egg yolk had significantly higher amounts of FAAs compared to egg white; however, after gastrointestinal digestion, the total FAA content of whole egg increased from 5.6 mg/g to 95.3 mg/g which is

around 17-fold raise (Figure 8.2). The total FAA content in boiled, fried and scrambled egg yolks increased 4.6-fold, 5-fold and 6.2-fold respectively (Figure 8.2).

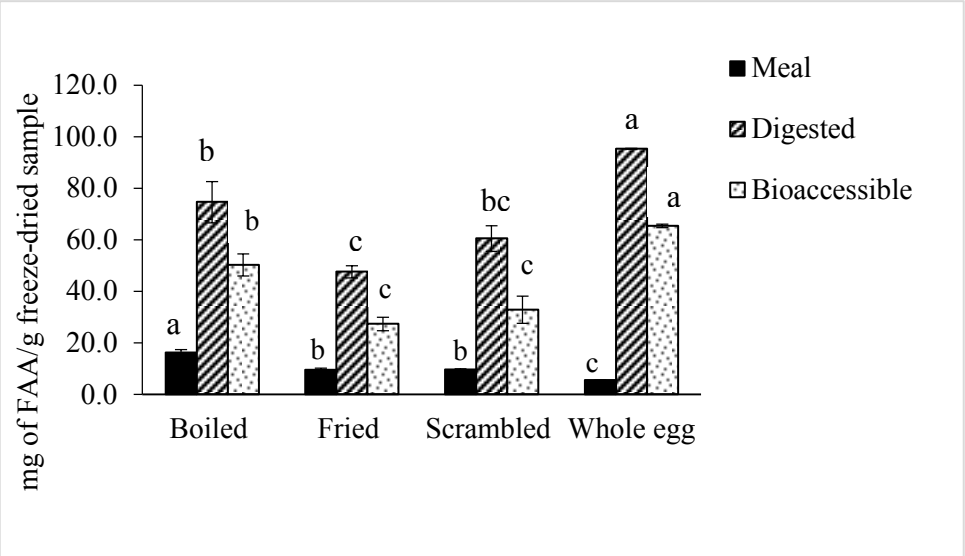

Figure 8.2. Total FAA content of cooked eggs before digestion (meal), after digestion (digested) and in jejunal and ileal filtrates (bioaccessible). [a-b] different letters denote significant difference (p <0.05) among the same type of sample i.e. meal, digested and bioaccessible in differently cooked eggs

Whole egg contains an approximately 12.3 g of proteins per 100 g of fresh sample, whereas in egg yolk the protein content is 16.1 g per 100 g fresh yolk (Seuss-Baum, 2007). The egg white proteins are easily broken down by intestinal proteases compared to egg yolk, where most proteins are bound to lipids (HDL and LDL) or associated with iron and phosphorous (phosvitin). After digestion, boiled yolk sample showed 74.6 mg of FAA per g sample, followed

by scrambled (60.5 mg/g) and fried (47.6 mg/g) egg yolks. You *et al*. reported that *in vitro* gastrointestinal digests of loach proteins contain 36.1% FAAs (You *et al*., 2010). Jensen *et al* also observed that simulated *in vitro* gastrointestinal digestion of saithe and shrimp increased the FAAs content after 75 min of digestion (Jensen, Abrahamsen, Maehre, & Elvevoll, 2009). Aromatic amino acids, tyrosine, tryotophan, histidine and phenylalanine are known to have antioxidant activities (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998; Christen, Peterhans, & Stocker, 1990; Gülçin, 2007; Pownall, Udenigwe, & Aluko, 2010). The total aromatic amino acid content of the undigested meal was approximately 15% for the yolk and 10% for the whole egg, but increased after digestion. In the digested sample, they made approximately 20% from the total FAA content. Although the total content of FAAs was lowest in fried sample, it had the highest percentage of total aromatic amino acids, which may partially explains the high antioxidant activity of fried sample (Figure 8.4). Our results are comparable with the studies of You *et al*. who observed that aromatic amino acids make 22% of FAAs in the loach protein digests (You *et al*., 2010). More than half of the total FAAs content was in the bioaccessible (ileal and jejunal) filtrates (Figure 8.2). Bioaccessible fraction of FAAs in boiled whole eggs and boiled egg yolk was similar with 68% and 67% from the total content of FAA respectively. Both fried and scrambled egg yolk had around 57% of total FAAs in the bioaccessible filtrates. Absorption of amino acids can depend on many factors such as the food matrix, distribution and composition of amino acid, kinetics of hydrolysis, removal of digestion products etc (Savoie, Agudelo, & Gauthier, 2005; Savoie, 1994). Over 70% of the total bioaccessible FAAs were absorbed during the first 150 min of the digestion (Figure 8.3). After that, passing of FAAs into the jejunal and ileal filtrates was minimal.

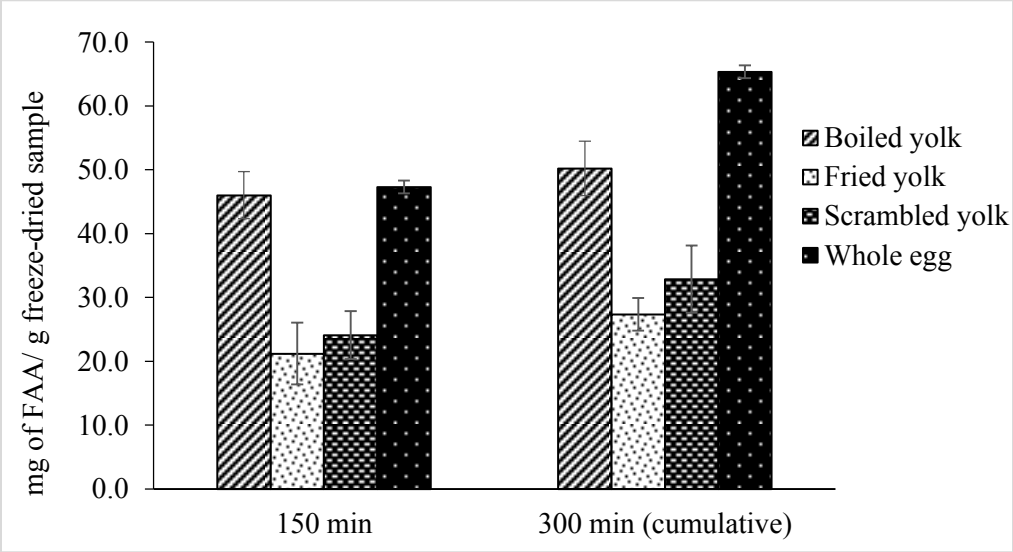

Figure 8.3. Cumulative quantity of bioaccessible FAAs at half time (0-150 min) and at the end of the digestion (0-300 min).

8.3.2. Effect of Digestion on Antioxidant Activity of Cooked Eggs.

In a complex food system, many different components can contribute to the total antioxidant activity. Among several chemical assays, ORAC assay has been considered as more suitable for estimation of the antioxidant activity of food related systems because of its capability of responding to a greater number of antioxidant compounds than other common assays (Clausen, Skibsted, & Stagsted, 2009; Ou, Chang, Huang, & Prior, 2013; Zulueta, Esteve, & Frígola, 2009). Since the digested eggs composed of a complex mixture of various compounds, ORAC assay was employed to determine the antioxidant activity. ORAC activity of all samples increased after digestion but in difference degrees (Figure 8.4). Fried egg yolk digests showed

the highest ORAC value of 425.8 ± 36.6 µmol TE/g, which is around 8-fold increment of the activity of cooked sample and scrambled egg yolk digests increased the activity up to 206.9 ± 33.8 µmol TE/g, a 5-fold increment. Similar results were reported by Jensen *et al*. who observed that 6-fold increase in the ORAC activity after gastrointestinal digestion of cooked pork and beef, compared to the undigested samples (Jensen, Dort, & Eilertsen, 2014). The ORAC values of pork and beef were approximately 400 µmol TE/g of dry meat (Jensen *et al*., 2014). Similarly, gastrointestinal digestion of shrimp and saithe increased the ORAC activity by 10-fold and 5-fold respectively (Jensen *et al*., 2009).

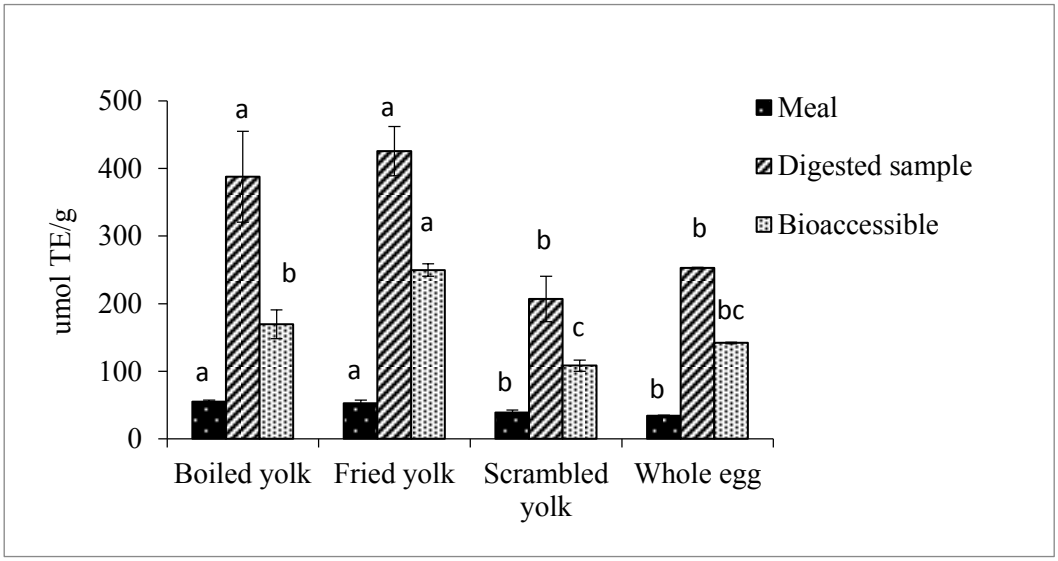

Figure 8.4. Changes of ORAC activity in eggs before and after digestion. [a-b] different letters denote significant difference (p <0.05) among the same type of sample i.e. meal, digested and bioaccessible in differently cooked eggs

Different cooking methods affected the ORAC activity of eggs (Figure 8.4). In cooked samples, antioxidant activity of boiled and fried egg yolks was higher than that of scrambled egg yolk and whole eggs. The same trend was observed after gastrointestinal digestion with boiled and fried

egg yolks showed the higher activity compared to scrambled yolk and whole boiled egg (Figure 8.4). ORAC activity of bioaccessible filtrates contributed approximately 45-55% to the total activity. Cumulative ORAC activity of bioaccessible filtrates at 150 min was doubled at the end of the digestion, at 300 min (Figure 8.5).

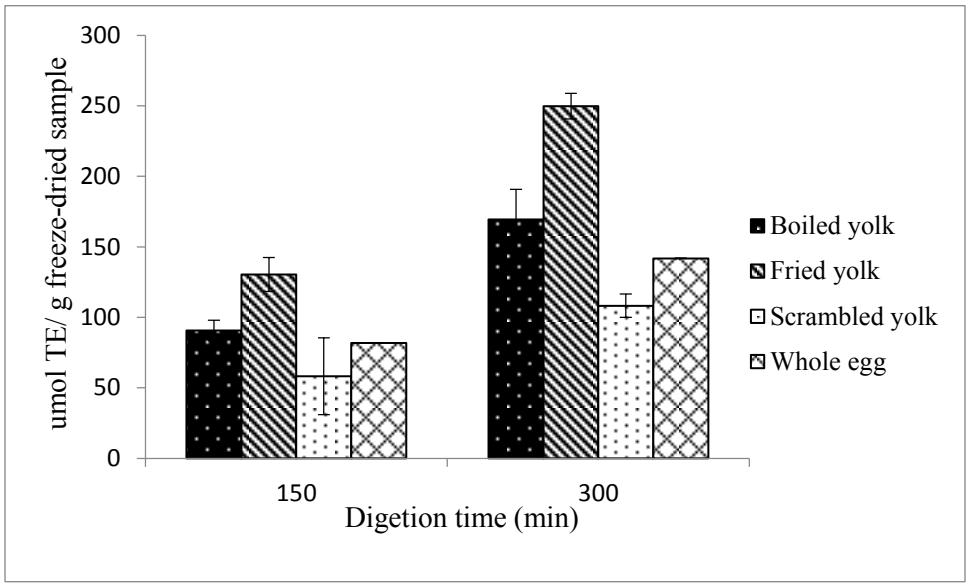

Figure 8.5. ORAC activity of bioaccessible portion (jejunal and ileal filtrates) after 150 min and after 300 min of digestion

In case of bioaccessible amino acids, after 150 min of the digestion, the amount passing to the jejunal and ileal filtrates was low. Many amino acids such as tyrosine, tryptophan, histidine, lysine, methionine, proline and glycine are known to have antioxidant properties (Marcuse 1960; Atmaca 2004). Several authors also observed that increased antioxidant activity after gastrointestinal digestion is associated with higher abundance of these amino acids in the digests (Jensen *et al*., 2009, 2014; Orsini Delgado, Tironi, & Añón, 2011; You *et al*., 2010). As shown in

Table 8.1, fried eggs had the highest percentage of aromatic amino acids from the total FAAs, which may partially explain the high ORAC activity of fried egg yolk.

Table 8.1 Amounts of aromatic amino acids (AAA) in before and after digestion of egg samples

	mg/ g meal undigested				mg/ g digested meal					Total FAA/ g digested meal	% of AAA from FAA
	Tyr	Trp	His	Phe	Tyr	Trp	His	Phe	Total		
Fried yolk	0.7	0.4	0.3	0.5	4.5	0.9	0.4	3.9	9.7	47.6	20.4%
Boiled yolk	1.2	0.2	0.4	0.5	7.2	1.0	1.1	5.5	14.8	74.6	19.8%
Scrambled yolk	0.7	0.1	0.5	0.9	6.0	1.0	0.8	4.2	12.0	60.5	19.8%
Whole egg	0.3	trace	0.1	1.3	9.0	1.6	1.5	6.9	19	95.3	19.9%

Egg is composed of many components with known antioxidant properties for example, proteins and peptides in egg white and FAAs, phospholipids, carotenoids, phosvitin, vitamins, minerals etc in egg yolk. During gastrointestinal digestion, the physicochemical nature of these compounds is greatly affected by the intestinal conditions generating an array of different compounds which may show antioxidant or pro-oxidant properties and have synergistic or antagonistic interactions with other compounds (Costa, Grevenstuk, Rosa da Costa, Gonçalves, & Romano, 2014; Gawlik-Dziki, 2012). In a previous study, we observed that FAAs, especially free aromatic amino acids, tryptophan and tyrosine are the major contributors to antioxidant activity of egg yolk (Nimalaratne *et al*., 2011). Although the FAAs increased after digestion,

they are not the major contributing factor to the antioxidant activity of digested egg. Generation of antioxidant peptides from egg white and egg yolk proteins after hydrolysis with digestive proteases is widely reported (Rao *et al*., 2012; Young, Nau, Pasco, & Mine, 2011). These peptides, may possibly contribute to the antioxidant activity of digested eggs (Dávalos *et al*., 2004). Therefore, further research is necessary to characterize these peptides. Apart from that other egg compounds such as carotenoids, phospholipids, vitamin E etc may also contribute.

In the present study we evaluated the effect of simulated gastrointestinal digestion on antioxidant activity of eggs using ORAC assay. Gastrointestinal digestion significantly increased the ORAC activity in egg yolk and whole egg samples. The free amino acid analysis revealed that digestion resulted in 17-fold increase in FAAs in whole egg and 4.5 to 6-fold in egg yolk. Different cooking methods resulted in variations of FAA content and antioxidant activity of eggs. Increase of FAA is not directly related to the increase of ORAC activity. Free aromatic amino acids contribute to the antioxidant activity, however was not the major contributing factor. Our results suggesting that antioxidant activity of eggs significantly increased upon gastrointestinal digestion and may offer beneficial effects on health.

CHAPTER 9 - Concluding Remarks

Eggs have long been viewed as the "natures' perfect food" due to the presence of many life-supportive nutrients. The perception of egg as a possible risk factor for heart diseases over the past several decades however has over weighted egg's inherent nutritive values, a consumer's shift that might have a devastating effect on the vulnerable egg industry, despite the fact that many human studies do not support an association between dietary cholesterol intake and risk of cardiovascular diseases (Djousse & Gaziano, 2008; Hu, 1999; Nakamura *et al*., 2007; Qureshi *et al*., 2007; Rong *et al*., 2013). The controversy over cholesterol and egg consumption is not be easily resolved, increasing evidence of egg for health research signifies the potential of egg as a health promoting food commodity rather than a food to be avoided. In addition to providing basic nutrients, current studies suggested many egg-derived beneficial activities such as antimicrobial, antihypertensive, antioxidant, and immunomodulating properties (Dávalos, Miguel, Bartolomé, & López-Fandiño, 2004; Hervé-Grépinet *et al*., 2010; Majumder *et al*., 2013; Miguel *et al*., 2008; Sava, 1996).

Many chronic diseases for example atherosclerosis, cancer, diabetes, aging, Alzheimer's disease and other degenerative diseases in humans (Finkel & Holbrook, 2000) are associated with oxidative stress, a condition caused by an imbalance between free radical formation and antioxidant defence (Valko *et al*., 2007). Antioxidants are believed to be effective in reducing oxidative stress. In comparison with extensive research on plant-derived antioxidants (Duthie & Crozier, 2000; Samaranayaka & Li-Chan, 2011), there is limited study on animal-derived antioxidants (Huopalahti, López-Fandiño, Anton, & Schade, 2007). The antioxidant activity of egg yolk compounds such as phospholipids (Sugino *et al*., 1997), carotenoids such as lutein and

zeaxanthin (Stahl & Sies, 2003) and phovitin (Xu, Katayama, & Mine, 2007) have been reported before. A hen is viewed as a "bioreactor" that may biologically transfer certain compounds from feed to the egg yolk. In addition to the well-known omega-3 fatty acids, phytochemicals such as soy isoflavone were also reported to transfer into egg yolk resulting increased antioxidant capacity (Saitoh, Sato, Harada, & Matsuda, 2004). Therefore, it is likely that phytochemicals presenting in feed might transfer into egg. Given the significance of oxidative stress to many kind of degenerative diseases including heart diseases, the main goal of this thesis was to establish the evidence that egg is an antioxidant commodity. For that we investigated the compounds contributing to egg antioxidant capacity and also evaluate the effects of storage conditions, cooking, and gastrointestinal digestion on egg yolk antioxidant activity.

The first study investigated the possibly accumulation of wheat and corn phytochemicals in egg yolk. As described in Chapter 3, egg yolk was initially applied to determine phenolic compounds by reversed-phase HPLC-DAD-MS/MS. Transfer of simple phenolic acids to egg yolk appeared to be minimal and only ferulic acid was detected in trace amounts in the MRM scan mode. Two aromatic amino acids, tryptophan and tyrosine were identified as the major contributors to the total antioxidant capacity of egg yolk extracts. The total antioxidant capacity was significantly affected by the method of cooking; microwave cooking caused the highest reduction in antioxidant capacity probably because of the degradation of antioxidative compounds due to high and uniform heat generation during microwave cooking.

Egg carotenoids, mainly lutein and zeaxanthin are known to accumulate in human eye retina and provide protection against oxidative damage and age-relating macular degeneration (AMD; Moeller, Jacques, & Blumberg, 2000). They are also known effective singlet oxygen scavengers and some studies suggested that they might play a protective role against heart

diseases (Ribaya-Mercado & Blumberg, 2004). Effects of cooking on egg carotenoids were studied (Chapter 4). Cooking of eggs caused reductions in all E-isomers and increased Z-isomers of carotenoids. All-*E*-lutein was the most affected, with 22.5%, 16.7% and 19.3% reduction in boiled, microwaved and fried yolk extracts respectively, while that of total xanthophyll loss ranged from 6 to 18%.

Although, the presence of antioxidants in egg yolk is well known, there is no systematic approach to extract total antioxidant compounds in egg yolk. We have developed a stepwise extraction method to extract egg yolk antioxidants (Chapter 5). The results showed that approximately two third of total antioxidant activity of egg yolk is due to free amino acids, followed by carotenoids, phosvitin and vitamin E.

Table eggs are stored in refrigerated temperature in Canada with a shelf life of approximately one month. We evaluated the effect of simulated retail storage conditions and cooking on antioxidant activity in normal table eggs, omega 3/lutein (n-3/lutein) enriched eggs and eggs from heritage chicken breeds (Chapter 6). Six weeks of storage at refrigerated temperature did not change the oxygen radical scavenging capacity (ORAC), the free amino content, carotenoid content or malondialdehyde (MDA) content in egg yolk. The ORAC value was the highest in n-3/lutein enriched eggs (161.4 μmol TE/g sample) and the lowest in eggs from heritage white leghorns (HW, 127.6 μmol TE/g sample). Boiling and frying significantly reduced the ORAC activity, free amino acid content, and lutein and zeaxanthin amounts and increased the MDA content in eggs.

Since egg yolk is eaten orally, simulated gastrointestinal digestion of egg yolk was performed to determine the effect of cooking and digestion on antioxidant activity and free amino acid content and egg carotenoid profile. After digestion, the antioxidant activity of fried

egg yolk increased from 53 to 426 μmol TE/g freeze dried egg yolk, which is around 8-fold increment (Chapter 7). In scrambled egg yolk the increment was around 5 times while in whole egg, it was 7 times. Egg carotenoids were stable during the digestion with average recoveries of 90% and 88% for all-*E* lutein and all-*E* zeaxanthin, respectively (Chapter 8). All-*E* lutein and all-*E* zeaxanthin from scrambled eggs showed significantly lower bioaccessibility compared to boiled eggs, which might be due to several factors including differences of fatty acid composition, particle size differences of the samples and chemical and structural changes of proteins and lipoproteins in egg yolk occurring during different cooking conditions.

In conclusion, this thesis demonstrated that egg is an antioxidant rich commodity due to the presence of aromatic amino acids, carotenoids, phosvitin and vitamin E. The antioxidant activity of egg yolk is stable during simulated storage conditions, decreased during domestic cooking, but was substantially increased by 5 to 8-fold after digestion. Simulated gastrointestinal digestion of eggs showed that egg carotenoids are stable during the digestion, whereas scrambling resulted in significantly lower bioaccessibility compared to boiled eggs. Although the content of free amino acids was increased 4 to 6-fold during digestion, the increase was not directly related to the increase of antioxidant activity, indicating the contribution of released antioxidant peptides, which remains to be revealed.

Overall, the results from this thesis greatly contribute to our understanding on antioxidant potential of eggs and their changes during storage, cooking and gastrointestinal digestion. The results from this thesis could be used by the egg industry to educate the general public and the consumers in regard to the potential health benefits of egg consumption as an antioxidant food commodity, which might promote egg consumption. The general public might increase their egg consumption or return to eat eggs in which contribute to a healthy balanced diet. However, it

should be noted that the antioxidant assays used in this thesis are based on *in vitro* chemical methods; hence there are limitations. *In vitro* chemical methods are extensively used to evaluate the antioxidant capacity of food products due to the simplicity, convenience and rapidity. Also, they would allow us to compare the activity of different food samples evaluated under same assay. However, these chemical assays may not necessarily reflect the conditions in the biological system. Therefore, the results obtained from chemical assays should be treated with cautious, as the activity may not be the same under *in vivo* conditions. The findings of this thesis provide the primary evidence of the role of egg yolk as an antioxidant food commodity. Therefore, further research studies will be needed to evaluate and establish these antioxidant benefits of eggs under *in vivo* conditions.

9.1 Recommendations for Future research

While the present research confirms *in vitro* antioxidant activity of egg yolk, further research is needed to validate the antioxidant effects *in vivo*. These studies can be started with suitable animal models and extended towards human clinical studies using appropriate biomarkers to assess the antioxidant properties. Recommended future studies are listed below.

1. It is important to perform animal model experiments using a suitable rodent model, to elucidate the antioxidant capacity of egg yolk and to understand the underlying mechanisms of antioxidant actions *in vivo.* Both healthy and disease models may be used under normal conditions and under oxidative stress in order to better understand the effects under different physiological status. The following tests/biomarkers may be used to evaluate the *in vivo* antioxidant effects.
    a. Biomarkers of oxidative damage

i. Biomarkers of lipid peroxidation – Levels of biomarkers such as hydroxyoctadecadienoic acid (HODE), F2-isoprostanes, neuroprostanes and malondialdehyde (MDA) in plasma

ii. Biomarkers of oxidative DNA damage - Urinary 8-hydroxydeoxyguanosine (8-OHdG)

iii. Oxygen radical absorbance capacity (ORAC) of plasma

b. Biomarkers of endogenous antioxidative defense system

i. Plasma coenzyme Q9 (CoQ9)

ii. Erythrocyte glutathione (GSH) levels

iii. Activity of endogenous antioxidant enzymes such as superoxide dismutase (SOD), thioredoxin reductase 1 (TXNRD1) and glutathione peroxide (GSH-Px) in tissue (brain, liver etc.) and plasma samples

2. Finally, human clinical trials will provide the most convincing information on actual bioavailability and bioactivities of egg yolk antioxidant compounds. These human studies should be carried out on healthy volunteers as well as on patients with various diseases. Following experiments may be used to evaluate the antioxidant effects.

a. Serum antioxidant status – ORAC assay, MDA content and cell-based antioxidant protection of erythrocytes (CAP-e) assay

b. Level of lipid peroxidation – total HODE content, cholesterol oxidation products (7α- and 7β-hydroperoxycholesterol (7α-OOHCh and 7β-OOHCh) in plasma, erythrocytes

c. Total thiols level of plasma

d. Activity of endogenous antioxidant enzymes (SOD, GPx etc.)

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