FATTY ACID PROFILE AND SENSORY CHARACTERISTICS OF TABLE EGGS PROCURED FROM HENS FED DESIGNER DIETS

By

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ABSTRACT

Omega-3 (n-3) enriched table eggs can serve as an important functional food to boost consumption of polyunsaturated fatty acids (PUFA) critical for good health. Because n-3 enriched eggs have the potential for unpleasant aromas and flavours, this research was designed to assess the fatty acid profile and sensory attributes of eggs procured from hens consuming designer diets. In the first study, diets containing hempseed oil (HO) and hempseed were tested. Hemp use in hen diets led to significant increases in n-3 PUFA content and colour intensity of egg yolks, but did not have adverse effects on the sensory profiles of the cooked eggs. Additionally, the level of docosahexaenoic acid (DHA) in eggs from the lowest HO group was the same as the highest HO group. In order to attempt to overcome this plateau, the second study assessed diets varying in linoleic acid (LA) content. Although docosapentaenoic acid in the yolk was significantly increased with an increasing dietary LA content, DHA remained unaffected by dietary treatment. In the third study, a different approach was used to reduce competition between ALA and LA. Diets containing two levels of alpha-linolenic acid (ALA) and varying ratios of saturated fatty acids (SFA): LA + oleic acid (OA) were tested. Increasing the SFA: LA + OA ratio resulted in marked increases in all n-3 PUFA. The fourth study was designed to assess the interaction between dietary constituents on sensory attributes of eggs, namely n-3 PUFA from flaxseed oil (FO), and canola meal (CM), which contains precursors to trimethylamine, which could possibly lead to fishy taint. Data from sensory panel descriptive analysis showed that oceanic flavour significantly increased with inclusion of FO, while egg, creamy and buttery flavours showed a decrease. The pairing of CM and FO resulted in a significant decrease in egg flavour compared to using FO alone. Overall, this research has

demonstrated that novel ingredients like hemp can be used in laying hen diets to deposit n-3 PUFA into eggs without fear of affecting sensory outcomes. In addition, increasing the ratio of SFA: LA + OA in layer diets is the most effective way to increase yolk ALA conversion into long-chain PUFA. The use of CM should be added with caution in layer diets when used in conjunction with n-3 PUFA ingredients to produce functional eggs due to a reduction in egg flavour. Future development of an egg yolk database will be useful for the egg industry in order to tailor specific hen diets that target specific compounds.

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DEDICATION

This thesis is dedicated to my family and friends, especially my mom, Kathryn and dad Marc, and my brother Michael. To Coco and Smoochie, thank you for your unconditional love through all our years. I would also like to dedicate this thesis to my Advisor Dr. Jim House for giving me the opportunity to work in his lab.

FOREWORD

This thesis was written in a manuscript format. The manuscript for the first study was published in the Journal of Food Science. The authors of this manuscript are Erin M. Goldberg[a], Naveen Gakhar[a], Donna Ryland[a], Michel Aliani[a], Robert A. Gibson[b] and James D. House[ac], Departments of [a]Human Nutritional Sciences & [c]Animal Science, University of Manitoba, Winnipeg, MB, R3T 2N2, Canada, [b]Department of Nutrition and Functional Food Science, University of Adelaide, Adelaide, South Australia 5000, Australia. The manuscript combining the second and third studies was published in Food Science and Nutrition with the same authors as above, with the exception of Naveen Gakhar. The manuscript for the fourth study has recently been submitted for publication, with the same authors as the second manuscript, with the exception of Robert Gibson.

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LIST OF ABBREVIATIONS

a*	red-green (scale +/-)
AA	arachidonic acid
ALA	alpha-linolenic acid
ANOVA	analysis of variance
b*	yellow-blue (scale +/-)
CCHS	Canadian Community Health Survey
CFIA	Canadian Food Inspection Agency
CHD	coronary heart disease
CM	canola meal
COX	cyclooxygenase
CVD	cardiovascular disease
D5DE	delta-5-desaturase
D6DE	delta-6-desaturase
DAG	diacylglycerides
DGLA	dihomo-gamma-1inolenic acid
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
DTA	docosatetraenoic acid
ELOVL2	elongation of very long chain fatty acid 2 gene
ELOVL5	elongation of very long chain fatty acid 5 gene
EPA	eicosapentaenoic acid
ETA	eicosatetraenoic acid
FA	fatty acid
FADS1	fatty acid desaturase 1
FADS2	fatty acid desaturase 2
FO	flaxseed oil
GLA	gamma-linolenic acid
HDL	high density lipoprotein

HO	hempseed oil
HPLC	high-performance liquid chromatography
HS	hempseed
IOM	Institute of Medicine
L*	lightness (scale 0-100)
LA	linoleic acid
LC	liquid chromatography
LCPUFA	long-chain polyunsaturated fatty acids
LDL	low density lipoprotein
LOX	lipoxygenase
LSD	least significant difference
LT	leukotrienes
lysoPE	lysophosphatidyl ethanolamine
lysoPL	lysophosphatidyl choline
MS	mass spectrometry
MUFA	monounsaturated fatty acids
n-3	omega-3
n-6	omega-6
NMR	nuclear magnetic resonance
NS	not significant
NSERC	Natural Sciences and Engineering Research Council of Canada
OA	oleic acid
PA	phosphoric acid
PALM	palmitic acid
PALMO	palmitoleic acid
PASW	statistical software
PC	phosphatidyl choline
PCK	rat model of polycystic kidney disease
PE	phosphatidyl ethanolamine
PG	prostaglandins
PL	phospholipids

PLS	partial least squares
PRC	prostacyclins
PUFA	polyunsaturated fatty acids
QTOF	quadrupole time-of-flight
SA	stearic acid
SD	standard deviation
SDA	stearidonic acid
SE	standard error of the mean
SFA	saturated fatty acids
SPSS	statistical software
TC	total cholesterol
TG	triglycerides
THA	tetracosahexaenoic acid
THC	tetrahydrocannabinol
TMA	trimethylamine
TMAO	trimethylamine oxide
TPA	tetracosapetaenoic acid
TTA	tetracosatetraenoic acid
TX	thromboxanes
VLDL	very low density lipoprotein

CHAPTER 1: GENERAL INTRODUCTION

Adequate consumption of omega-3 polyunsaturated fatty acids (n-3 PUFA) has been shown to have a beneficial impact on indices of cardiovascular disease (CVD), cancer, inflammatory disease, brain function and mental health (Erasmus 1993; Ruxton and others 2004; Ruxton and Derbyshire 2009). Unfortunately, consumption of such lipids in Western diets is not adequate, especially for reasons related to the unacceptable sensory properties of fish, and concerns over heavy metal contamination and unsustainability (Farrell, 1998; Bays 2007). A recent study assessing the diets of pregnant Canadians found that their mean n-3 PUFA intake was $0.57 \pm 0.06\%$ of energy, just below the minimum target of 0.6% of energy, and 65% of women fell below the Acceptable Macronutrient Distribution Range (Denomme and others 2005). Their mean DHA intake was 82 ± 33 mg/d, with 90% of the women consuming less than 300 mg/d. Results from the 2004 Canadian Community Health Survey (CCHS) showed that Canadian men and women had median ALA intakes exceeding the Adequate Intakes of 1.6 g/d and 1.1 g/d, respectively. Similar findings were observed in an analysis using observational data from the National Health and Nutrition Examination Survey between 2003-2008 which revealed that U.S. adults consume a median intake of 1.6 ± 0.04 g/d ALA. However, median intakes of only 41 ± 4 mg/d EPA and 72 ± 4 mg/d DHA were observed (Papanikolaou and others 2014). The Institute of Medicine (IOM) recommends 160 mg/d and 90 mg/d of total n-3 LCPUFA for men and women, respectively. However, based on epidemiological findings showing a beneficial role of n-3 LCPUFA in the prevention of cardiovascular and inflammatory diseases, suggested dietary targets of 610 mg/d and 430 mg/d are recommended for reducing chronic disease risk. With North Americans not reaching desired intake levels, it is clear that strategies

to increase consumption need to be developed. There are a number of ways consumption can be increased by circumventing the need to consume fish, including the deposition of these important fatty acids into regularly consumed foods, called functional foods.

One such functional food is n-3 enriched table eggs, which can be procured from hens fed diets containing these lipids. The hen liver has the capacity to convert alpha-linolenic acid (ALA), the parent compound of the n-3 PUFA series found in plant sources, into long-chain polyunsaturated fatty acids (LCPUFA) like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) found abundantly in fish. Eggs in general have been consumed for centuries, and contain all of the ingredients for a new life, including lipids, amino acids and an array of vitamins and minerals (Farrell, 1998). The egg industry in Canada has already been fortifying eggs with n-3 PUFA for decades, and these eggs account for approximately 15% of the egg market space (Flax Council of Canada 2014). Enriched egg consumption has been on the rise and greatly appeals to consumers, whose demand for "natural" products with health-promoting properties increases. In fact, consumption of n-3 enriched eggs has been reported to lower blood pressure and plasma triglycerides (TG) in humans, which has been associated with a reduced risk of coronary heart disease (CHD) (Oh and others 1991; Ferrier and others 1992; Ferrier and others 1995).

Current industry practice of producing enriched eggs involves feeding either flaxseed, or flaxseed combined with a small amount of fish oil. But there are three main problems with these strategies: 1) heavy reliance on flaxseed could be cause for concern if market or growing conditions are not ideal which may drive up its cost, 2) fish oil is expensive and vegetarian consumers may not accept it as a feed ingredient and 3) aroma and/or flavour profiles of enriched eggs that deviate from conventional eggs negatively impact acceptability and purchase behavior

among consumers, potentially negating the goal of increasing consumption. Also, the fatty acid composition of the egg yolk should be optimized in order to deposit the most LCPUFA possible.

Not only do novel sources of n-3 PUFA need to be identified and tested for safety and efficacy in layer diets, but other strategies to optimize the fatty acid composition of eggs need to be elucidated in order to maximize the health benefits of consuming enriched eggs. Also, current production practices do not necessarily take dietary interactions into account, and these may impact both the deposition of fatty acids into eggs and the respective sensory quality.

For this thesis, Chapter 2 summarizes the current literature. In Chapter 3, the hypotheses and objectives of this research are outlined. In Chapter 4 the impact of feeding hemp products to hens is presented. Chapter 5 discusses the use of diets containing oils designed to maximize ALA conversion into LCPUFA. Chapter 6 presents data on the interactions between the inclusion of canola meal (CM) and flaxseed oil (FO) in the hen diet in relation to egg sensory outcomes. Finally, Chapters 7, 8 and 9 provide a general discussion, an overall summary of the data and considerations for future research directions, respectively.

CHAPTER 2: LITERATURE REVIEW

2.1. Fatty acid nutrition and metabolism

Lipids are a diverse and ubiquitous group of compounds that play a role in numerous biological functions, including acting as structural components of cell membranes and increasing their fluidity (Fahy and others 2011). They also serve as an energy storage source and participate in key signaling pathways and can be considered extremely important for survival and overall health and wellbeing. Essential nutrients cannot be synthesized by the body, and therefore must be obtained through the diet. There are about 50 essential nutrients necessary for human health, including two fatty acids; LA and ALA. Lipids are divided into 8 categories; fatty acids, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, polyketides, sterols and prenols (Fahy and others 2009).

Fatty acids are composed of a hydrocarbon chain that terminates with a carboxylic acid group, and may also be attached to functional groups containing oxygen, halogens, nitrogen and sulfur. They are further divided into saturated and unsaturated fatty acids according to their chemical structure and unsaturated fatty acids can be further divided into either monounsaturated fatty acids (MUFA) or PUFA depending on the number of double bonds present in the molecule (Bezard and others 1994). Depending on the location of the first double bond relative to the methyl terminus, they can be classified as either n-6 or n-3 (Anderson and Ma 2009). However, there are others, including those from the n-9, n-7 and n-4 families. Key PUFA chemical structure names can be found in Table 2.1.

Table 2.1

Lipid names of major n-3 and n-6 fatty acids

Omega-3		**Omega-6**	
Common Name	*Lipid Name*	*Common Name*	*Lipid Name*
Alpha-linolenic acid (ALA)	18:3n3	Linoleic acid (LA)	18:2n6
Stearidonic acid (SDA)	18:4n3	Gamma-linolenic acid (GLA)	18:3n6
Eicosapentaenoic acid (EPA)	20:5n3	Arachidonic acid (AA)	20:4n6
Docosapentaenoic acid (DPA)	22:5n3		
Docosahexaenoic acid (DHA)	22:6n3		

(AOCS Lipid Library 2014)

2.1.1. Metabolism of essential fatty acids

ALA is the parent compound of the n-3 PUFA series, and the most common n-3 fatty acid found in terrestrial diets (Brenna 2002). There are 3 metabolic fates of ALA when it is consumed. The first is its desaturation (addition of a double bond) by delta-6-desaturase enzyme (D6DE) to form stearidonic acid (SDA), and further elongation (addition of 2 carbon atoms) and desaturation to be converted into LCPUFA like EPA, DPA and DHA. The other possibility is β-oxidation, with the shunting of resulting acetate to complete oxidation to CO_2 *or* using acetate for biosynthesis (Burdge 2006). LA is the parent compound of the n-6 PUFA series and most commonly found in vegetable oils. When LA is ingested, it becomes desaturated into gamma-linolenic acid (GLA) followed by elongation, to give dihomo-gamma-linolenic acid (DGLA) (Furuse and others 1991). Dihomo-gamma-linolenic acid forms AA by a further hydrogenation, and can continue on into docosatetraenoic acid, then the n-6 DPA through subsequent elongation-desaturation reactions.

Efficiency of ALA conversion to LCPUFA in humans is quite low, estimated to be 5-10% to EPA and 2-5% for full conversion to DHA (Arterburn, Hall, Oken 2006; Plourde and Cunnane 2007). The International Society for the Study of Fatty Acids and Lipids (ISSFAL)

recently concluded that infants can convert approximately 1% of dietary ALA into DHA, while in adults the value is almost negligible (<0.1%) (Plourde and Cunnane 2007; Brenna and others 2009). If demands for n-3 PUFA are modest and primarily serve to support membrane turnover and renewal in adults, then it may be possible that this limited capacity to convert ALA into LCPUFA may be sufficient to maintain tissue function in healthy individuals consuming a balanced diet (Burdge 2004). However, for individuals with diabetes or other metabolic disorders where conversion enzymes may be compromised, and in those who have inherited a limited ability to produce these enzymes, maintaining adequate n-3 PUFA status is important (Simopoulos 1999).

ALA conversion to LCPUFA can be downregulated due to a high consumption of n-3 LCPUFA (Cunnane 2003; Gibson and others 2013), LA (Emken and others 1993; Brenna 2002), saturated fat and trans fat in the diet. In addition, various lifestyle factors including smoking, alcohol consumption, stress, and magnesium deficiencies may also impact ALA conversion (Mahfouz and Kummerow 1989; Marangoni and others 2004; Ghezzi and others 2007). Stable isotope tracer studies have also indicated that ALA conversion occurs to a greater extent in women than in men, a 2.5 fold greater rate (Burdge and Calder 2005). Young women have been found to convert up to 21% of ALA into EPA (Burdge and Wootton 2002). In fact, one study found that administration of oral ethinyl estradiol in female-to-male transsexual subjects increased DHA by $42 \pm 8\%$ (P<0.0005), whereas transdermal 17β-estradiol had no effect (Giltay and others 2004). Additionally, parenteral testosterone decreased DHA by $22 \pm 4\%$ (P<0.0005). The authors proposed that hepatic synthesis of DHA represents the major source of the increase in DHA in women compared with men, and estrogen plays an important role in upregulating the synthesis of DHA from ALA. Age differences have also been observed. The proportion of

labeled ALA converted to EPA and DPA, estimated from the relative time vs. concentration curves of individual labeled fatty acids in plasma was between three- and six-fold lower in older individuals (Burdge and Wootton 2002; Burdge, Jones, Wootton 2002; Burdge and others 2003). This suggests that older men are less able to convert ALA to these longer-chain PUFA, and may need to consume preformed LCPUFA to maintain DHA status. However, DHA synthesis did not appear to differ between these groups, although the level of incorporation of label into DHA may have been too low for any differences to be detected. Unfortunately, age studies have not been conducted in women so it is unknown whether increasing age yields the same effect as in their male counterparts.

Differences among individuals of the same background have also been observed. In a cohort of 727 Caucasian adults, it was found that carriers of the minor alleles of 11 single nucleotide polymorphisms (SNPs) (rs174544, rs174553, rs174556, rs174561, rs3834458, rs968567, rs99780, rs174570, rs2072114, rs174583 and rs174589) found in the FADS1 and FADS2 gene cluster, had higher serum phospholipid contents of LA and ALA, and lower levels of AA, EPA and DPA (Glaser and others 2011).

2.1.2. Oxylipin production

Oxylipins are a class of highly bioactive fatty acid metabolites that include the octadecanoids, eicosanoids, and docosanoids. The first step in their formation is the release of AA from membrane PL by phospholipase A_2, followed by the formation of prostanoids via the cyclooxygenase (COX) pathway, mono-, di- and tri-hydroxyeicosatetraenoic (HETE) acids and leukotrienes (LT) via the lipoxygenase (LOX) pathway, and epoxyeicosatrienoic acids and HETE via the cytochrome p450 pathway (Devassy and others 2013; Ibrahim and others 2014). There are two isozymes of COX (responsible for converting AA into prostaglandins) encoded by

distinct gene products: COX-1 and COX-2, which differ in their regulation of expression and tissue distribution (Husain and others 2002). COX-1 is expressed in the gastrointestinal tract whereas COX-2 at sites of inflammation (Hawkey 2001). The AA-derived 2-series prostaglandins (PG), prostacyclins (PRC), thromboxanes (TX) and 4-series LT can promote inflammation, vasoconstriction and platelet aggregation (James, Gibson, Cleland 2000). Alternatively, the EPA-derived 3-series PG, PRC, TX and 5-series LT are much less inflammatory. In a recent randomized controlled trial, participants with peripheral arterial disease consuming 30 g milled flaxseed per day for 6 months had significant decreases in plasma oxylipins compared to the control (Caligiuri and others 2014). Six of these oxylipins were products of soluble epoxide hydrolase, and patients whose levels of these oxylipins were decreased also exhibited significant decreases in systolic blood pressure (mean [95% confidence interval], -7.97 [-14.4 to -1.50] mmHg versus +3.17 [-4.78 to 11.13] mmHg). In another study where a targeted lipidomic analysis of oxylipins were performed using kidneys from normal and PCK rats with renal disease, heat map analyses of ALA oxylipins were found to be elevated in FO feeding while LA oxylipins were decreased with FO and fish oil compared to a soy oil control. Fish oil feeding resulted in the greatest increase in DHA oxylipins, but both FO and fish oil resulted in EPA elevations. Therefore, increasing dietary n-6 PUFA contributes to competition with n-3 PUFA for the COX and LOX enzymes, leading to antagonistic action in physiological functions, which would result in a strongly pro-inflammatory effect in the body. Increasing dietary n-3 PUFA would have the opposite effect in the body. This explains why the dietary ratio of n-6:n-3 PUFA is important in maintaining good health.

2.1.3. Proper balance for optimal health

The agricultural revolution resulted in the modification of dietary patterns over the last 200 years. With the efficiency of grain and vegetable oil processing, the consumption of n-6 PUFA increased significantly in the form of processed and refined foods whereas there was a marked reduction in whole food consumption including fatty fish, which reduced n-3 PUFA intake (Antruejo and others 2011; Gómez Candela, Bermejo López, Loria Kohen 2011). The general recommendations for essential fatty acid consumption in adults range for LA and ALA at 5-10 and 0.6-1.2% (as a percent of total energy), respectively (Health Canada 2012). It is also recommended that up to 10% of the acceptable macronutrient distribution range for n-3 PUFA be consumed in the form of EPA and/or DHA. The IOM recommends a n-6:n-3 ratio between 5:1 and 10:1, but Canada's Scientific Review Committee (1990) determined that Canadians should aim for a daily n-6:n-3 ratio roughly between 5:1 and 6:1 for "optimal health", because these proportions do not promote an over-accumulation of metabolic intermediates that would hinder fatty acid metabolism (Leizer and others 2000). For instance, competition by EPA results in decreased production of TXA_2, LTB_4, and PGE_2 metabolites, which reduces platelet aggregation, vasoconstriction, leukocyte chemotaxis and adherence (Simopoulos 2002). The metabolism of EPA results in less potent eicosanoids (Das 2006). A concurrent rise in TXA_3, prostacyclin PGI_3 and LTB_5 occurs, inhibiting platelet aggregation and vasoconstriction, and promoting vasodilation (Simopoulos 2002). This results in a reduction of atherosclerotic plaques by making blood less viscous, blood flow with reduced resistance, thus contributing to beneficial outcomes related to CVD. But it has been suggested that the optimal ratio should be even lower, ranging from 1:1 to 5:1 (Simopoulos 1999; Holub 2002). One study found that a ratio of 4:1 allows for adequate conversion to DHA in healthy vegetarians, while another study suggested that 2.3:1 was optimal (Indu and Ghafoorunissa 1992; Masters 1996). In the 1990's, most North

Americans regularly consumed a ratio between 10:1 and 30:1 (Simopoulos 1999), which is an extreme deviation from either recommendation. More recent findings from CCHS have found that the n-6:n-3 ratio in adults' diets fell between 7:1 and 8:1, depending on the age and sex group (CCHS 2.2 2004).

2.1.4. Health benefits of consuming fatty acids of interest

An n-3 fatty acid deficiency can lead to skin diseases, heart disease, inflammatory conditions in addition to premature aging and central nervous system disorders. However, the health benefits of consuming a diet rich in these PUFA was not known until the 1980's. Early observations of the northern Inuit were such that they had a low incidence of heart disease despite a diet high in total fat (Horrobin 1987). It was found that the deep-water fish they consumed were rich in n-3 LCPUFA (Kromhout, Bosschieter, Coulander 1985). Subsequent research was undertaken to determine the various health effects related to n-3 consumption, and it was demonstrated that consuming a diet rich in such PUFA was associated with the prevention or management of heart disease, cancer, arthritis, diabetes, depression, stroke, asthma and hypertension. Adequate consumption has also been found to delay the loss of immunological functions and promotes infant brain development, visual development, healthy skin, shiny hair and weight management (Neuringer, Anderson, Connor 1988; Erasmus 1993; Temple 1996; Pandalai and others 1996; Rose 1997; Leizer and others 2000; Laca, Paredes, Díaz 2009). N-3 PUFA have been found to normalize blood pressure, blood cholesterol levels and fat metabolism, decrease insulin dependence, and increase metabolic rate and membrane fluidity (Deckelbaum and Torrejon 2012). Of all the n-3 PUFA, it is suggested that DHA has the most important biological effect as it contributes to the fluidity in cell membranes, necessary for optimal development of the nervous system and is especially abundant in neural and retinal tissue

(Cherian and Sim 1992). For that reason, DHA is considered to be essential in visual and neurological development, particularly in premature infants (Neuringer, Anderson, Connor 1988). Despite inefficient conversion, there are potential roles in human health for ALA and EPA independent of their metabolism to DHA. There are conflicting reports in the literature on the benefits of various n-3 PUFA. Recent results from a systematic review showed that n-3 LCPUFA from marine sources, administered either in food or in supplement form for at least 6 months, reduces cardiovascular events by 10%, cardiac death by 9% and coronary events by 18% (Delgado-Lista 2012). Another systematic review and meta analysis of both dietary and biomarker studies suggested that ALA consumption is associated with a moderately lower risk of CVD (Pan and others 2012). On the other hand, a different study showed that dietary and circulating levels of EPA and DHA, but not ALA, were inversely associated with CVD incidence (de Oliveira Otto and others 2013). Overall, the evidence supporting the health benefits of ALA appears to be less convincing since it hasn't been studied as extensively as DHA. It is also unclear as to whether or not ALA has beneficial effects beyond conversion to LCPUFA (Harris and others 2008). In one study, it was found that short-term ALA supplementation in healthy elderly subjects improved concentrations of LDL cholesterol and apolipoprotein B more favourably than EPA and DHA (Goyens and Mensink 2006).

2.2. Food sources of n-3 polyunsaturated fatty acids

2.2.1. Plant sources

Table 2.2 displays the n-3 fatty acid content of some common food sources. N-3 PUFA can be produced by various plants, including canola, soybean, walnuts, and wheatgrass (Harris and others 2009). Flax, a crop grown abundantly on the Canadian prairies which contains

upwards of 41% lipid in the whole seed, is one of the richest sources of n-3 PUFA, with 18%

ALA present in the whole seed, and 56% in the seed oil (Scheideler and Froning 1996). The 25-

35% oil that is contained within whole HS consists of more than 90% unsaturated fatty acids,

with LA at around 57% and ALA at 17-19% (Oomah and others 2002). Less common sources of

n-3 PUFA include camelina (*Camelina sativa L.Crantz)*, perilla, kiwi, chia, at 35, 58, 62 and

64% ALA in the seed oil, respectively (Zubr 1997). Pearl millet is rich in oil compared to

common cereals, having a typical fat content above 5%, with ALA comprising 4% of the total

fatty acids (Rooney 1978; Collins and others 1997). Although the chloroplast membranes of

green leafy plants are very rich in n-3s, the amount as a whole food is modest (Bezard and others

1994). Smaller amounts can also be found in legumes and cauliflower.

Marine microalgae is the original source of n-3 PUFA in the diet of fish, containing high

levels of DHA via fermentation (Barclay and others 1994; Laca, Paredes, Díaz 2009). In fact,

marine algae contains approximately 3.8% DPA and 7.4% DHA on a dry weight basis, but no

EPA (Herber and Van Elswyk 1996).

2.2.2. Animal sources

LCPUFA are not found in plants, but are ubiquitous in mammalian tissue, achieving

highest concentrations in the membranes of neural and other excitable tissue (Brenna and Diau

2007). The richest animal source of n-3 PUFA, mainly in the form of EPA and DHA, includes

fatty fish such as salmon, tuna, mackerel, herring, trout and sardines (Simopoulos 1991).

Crustaceans and mollusks also contain a small amount. Between fish species there are large

variations in n-3 PUFA content, with some containing substantially higher levels of EPA

compared to DHA and vice versa. For example, pink salmon canned in water (100 g) may

contain 1665 mg LCPUFA, while the same amount of canned tuna contains only 229 mg (Meyer

2011). Other minor sources include dairy, eggs, chicken, pork and beef. However, the amount

of n-3 PUFA in these foods is substantially lower than in fish, at times negligible, and highly

variable depending on the diet of the animal.

Despite a high content of n-3 LCPUFA, a limitation of using fish oils as a feed ingredient

in poultry diets is not only cost, but also safety. Many potentially toxic pollutants are fat soluble,

and feeding fish oil to layers may result in increased elevations of such marine pollutants in

subsequent egg yolk (Domingo 2014). Furthermore, fish consumption advisories meant to

protect human health do not extend to fish products fed to farmed animals such as laying hens

(Dórea 2006). In Canada and the United States, approximately 20% of stocks are overfished

which leads to disrupted ecosystems (National Research Council 2013).

Table 2.2

Omega-3 fatty acid content of various plant and fish oils

	% ALA	*% EPA*	*% DPA*	*% DHA*
Plant oils				
Flax	56	0	0	0
Hemp	18	0	0	0
Walnut	12	0	0	0
Canola	9	0	0	0
Soybean	7	0	0	0
Corn	1	0	0	0
Fish oils				
Salmon	1	13	3	18[a]
Sardine	1	10	2	11[a]
Cod liver	1	7	1	11[a]
Menhaden	1	13	5	9[a]
Herring	1	6	1	4[a]

(Canadian Nutrient File 2014; USDA Nutrient Database 2014[a])

2.2.3. Functional foods

Functional foods are natural or processed foods that contain known biologically active compounds that can deliver a clinically proven health benefit, thus aiding in the prevention, management and treatment of chronic diseases (Arvanitoyannis and Van Houwelingen-Koukaliaroglou 2005). Dietary consumption of marine products is low, especially in North America, so other sources of n-3 LCPUFA need to be developed in order for adult men and women to reach desired intake levels (Laca, Paredes, Díaz 2009). The most direct way to increase consumption of LCPUFA is through the microencapsulation of fish oil into standard foods (Rymer and Givens 2005; Kolanowski and Laufenberg 2006). However, this method is expensive, not environmentally sustainable due to overfishing and may contain significant levels of contaminants like methylmercury, polychlorinated biphenyls or dioxins (Falcó and others 2006; Domingo and Bocio 2007). Also, oil derived from farmed fish may contain significantly less n-3 PUFA compared to their wild counterparts because of the feed utilized. The alternative method is to alter the fatty acid composition of regularly consumed foods that make a significant contribution to fat intake (Rymer and Givens 2005), as this may be the best long-term solution to boost intake of total n−3 PUFA (Molendi-Coste, Legry, Leclercq 2011).

One way this can be achieved is through the creation of functional foods like n-3 eggs, achieved by altering the diet of the laying hen (Beynen 2004). Eggs have been collected and consumed for centuries before the domestication of hens and other birds (Rose 1997). They are a good choice for n-3 enrichment because eggs are highly palatable to a wide range of people across all cultures (Surai and Sparks 2001). Since 1960, global egg production has increased by nearly four times (Speedy 2003). Annual per capita consumption of eggs in Canada and the United States in 2009 was 12 kg and 14 kg, respectively (FAO 2009). A joint IFPRI/FAO/ILRI study suggested that global production and consumption of eggs will continue to rise by 30% by

the year 2020 (Delgado and others 1999). In addition, eggs are inexpensive to produce, highly

nutritional, and can be used for a variety of functions in cookery. Because the hen's liver

reduces contaminants in the diet, n-3-enriched eggs can be considered a safer alternative to fish

and other marine sources (Farrell 1998; Bays 2007). A recent review concluded that the benefits

of regularly consuming eggs by the general population clearly outweigh the potential health risks

derived from potential exposure to a number of chemical pollutants through that consumption

(Domingo 2014). Furthermore, setting aside safety concerns, many people find it more practical

to increase egg consumption rather than fish consumption because of taste and versatility.

2.3. Production of n-3 enriched table eggs

2.3.1. Egg composition

An egg contains all the essential nutrients, including lipids, amino acids and vitamins,

required for a new life (Farrell 1998). Hen eggs consist of a protective porous shell (0.2-0.4 mm

thick), albumen (white), and vitellus (yolk), contained within various thin membranes. The egg

yolk is surrounded by the egg white, which is an aqueous, gel-like liquid that is mostly composed

of water and protein. The yolk, which is suspended in the center of the egg by the chalazae, is the

part of an egg that feeds the developing embryo, and contains most of the nutrients. In fact, all

of the n-3 PUFA in eggs are contained within the yolk. When the hen's diet is altered, it is the

yolk's composition that is affected, not the albumen.

The lipid fractions of egg yolk can be found in Table 2.3. The average egg contains 6

grams of lipid in the form of a) TG (66%), esters derived from glycerol and three fatty acids, b)

PL (30%), major components of all cell membrane as they form PL bilayers, most of which

contain a diglyceride, a phosphate group and a simple organic molecule and c) free cholesterol

(5%), a waxy steroid of fat manufactured in the liver or intestines. Other minor lipid components

consist of cholesterol esters and free fatty acids (Rose 1997).

Table 2.3

Lipid fractions of egg yolk

Lipid fraction	% of total lipids
Triglycerides	66
Phospholipids	28
Phosphatidyl choline (PC)	73
Phosphatidyl ethanolamine (PE)	15.5
Lysophosphatidyl choline	5.8
Sphingomyelin	2.5
Lysophosphatidyl ethanolamine	2.1
Plasmalogen	0.9
Phosphatidyl inositol	0.6
Cholesterol, cholesterol esters	5
Carotenoids, xanthophylls	1

Table adapted from (Belitz, Grosch, Schieberle 2009; Rhodes and Lea 1957)

Similar to humans, the chicken's liver is the primary site for FA synthesis (Leveille and

others 1975). Yolk lipid composition is the result of a combination of *de novo* lipogenesis and

the transfer of lipid components from the diet. Upon absorption in the intestinal lumen,

hydrolyzed products of lipid digestion such as long chain fatty acids and monoacylglyerols must

be re-esterified within the endoplasmic reticulum of enterocytes prior to transport

(Yannakopoulos 2007). The resultant TG are packaged with cholesterol, PL and proteins to form

lipoproteins called portomicrons, instead of chylomicrons found in humans, because they are

transferred to the hepatic portal circulation. Short chain fatty acids and free glycerol are

transported in this manner as well. The lipids reform and are included in particles of very low

density lipoprotein (VLDL), passing to the Golgi apparatus where they acquire PL and further

glycosylation. Finally, completed particles of VLDL concentrate in secretory vesicles and are then discharged into the blood. The VLDL are then carried by the blood to the ovarian follicles where they diffuse through the holes in the capillaries. Particles enter the yolk via receptor-mediated endocytosis through the oolemma. Typically, ALA is in the form of TG, and LCPUFA as PL in egg yolk (Scheideler and Froning 1996).

Conventional hen diets result in eggs with a n-6:n-3 ratio of approximately 13:1, much higher than recommended for optimal health as described earlier (Scheideler and Froning 1996). However, an enriched egg can provide upwards of 500 mg total n-3 PUFA, which may contain up to 290 mg of combined EPA and DHA. This could provide adults with half of the necessary recommendation (500 mg EPA and DHA per day) to reduce the risk for developing CVD (Harris and others 2008).

Table 2.4

Comparison of the nutrient content of enriched and conventional eggs[1]

Nutrient	Omega-3 Egg	Conventional Egg[2]
Energy (calories)	70	70
Protein (g)	6.0	6.0
Total Fat (g)	5.0	5.0
Saturated Fat (g)	1.5	1.5
Monounsaturated Fat (g)	2.0	2.0
Total Omega-6 Fatty Acids (g)	0.8	0.6
Linoleic acid (mg)	640[5]	540
Arachidonic acid (mg)	30[5]	80
Total Omega-3 Fatty Acids (g)	0.4[3]	0.1
Alpha-linolenic acid (mg)	174[5]	31
Eicosapentaenoic acid (mg)	17[5]	1
Docosapentaenoic acid (mg)	10[5]	4
Docosahexaenoic acid (mg)	108[5]	34[4]
Cholesterol (mg)	195	195

[1]Abbreviations: g = grams, mg = milligrams.

[2]Nutrient content for 1 large hard-boiled chicken egg, (Canadian Nutrient File 2014)
[3]On average, a commercially available n-3-enriched egg contains about 75 mg DHA for an egg from a flax-fed hen, and 125 mg DHA from a hen fed both flaxseed and fish oil (Burnbrae Farms 2014).
[4]Taber and others 1998
[5]Gillingham and others 2005

Flax incorporation at 10% or 20% of a poultry ration can increase the ALA content of egg yolk fat from 0.4% in the conventional egg to 4.6% and 8.9%, respectively (Flax Council of Canada 2014). Table 2.4 shows the composition of n-3 enriched eggs in Canada.

2.3.2. Factors affecting yolk lipid composition

Hens are able to synthesize n-3 PUFA from short chain fatty acids in the diets by carbon chain elongation and desaturation (Gonzalez-Esquerra and Leeson 2001), and deposit these substances into the yolk (Mennicken and others 2005). The enrichment of the egg yolk with LC-PUFA can occur through two main pathways; the direct deposit of these fatty acids from the diet (ie. preformed DHA) and as a final synthesis from its primary precursor (ie. ALA) through the synthesis of the intermediary products, a result of the *de novo* synthesis (Leskanich and Noble 1997; Baucells and others 2000; Yalçyn, Kemal Ünal, Basmacyoolu 2007). Overall, the conversion efficiency from ALA to DHA is largely dependent on the total amount of n−3 PUFA in the diet, as well as on their relative proportions (Cachaldora and others 2008b).

As depicted in Figure 2.2, the essential fatty acid precursors in the n-6 (LA) and n-3 (ALA) pathway are converted into fatty acids of longer chain length and a higher degree of unsaturation within the liver by alternating desaturation and chain elongation reactions (Sprecher 1981). The key desaturase enzymes, delta-5 (D5DE) and D6DE are encoded by the genes fatty acid desaturase 1 and 2 (FADS1 and FADS2, respectively) (de Schrijver and Privett 1982; Cho, Nakamura, Clarke 1999; Schaeffer and others 2006).   The genes elongation of very long chain fatty acid 2 (ELOVL2) and elongation of very long chain fatty acid 5 (ELOVL5) are responsible

for the elongation reactions (Gregory and others 2013). Desaturation adds a double bond by removal of hydrogen, which is catalyzed by delta-desaturase whereas elongation reactions add two carbon atoms. These pathways are well established and observed across kingdoms (Nakamura and Nara 2004).

More specifically, ALA is first desaturated to SDA by D6DE. This is followed by elongation to DGLA by an elongase, which is then desaturated to form EPA. In chickens, EPA is elongated and desaturated and the further elongation of DPA to tetracopentaenoic acid involves the combined effect of ELOVL5 plus ELOVL2, enabling a better efficiency of conversion than any other species (Gregory and others 2013). Furthermore, C24:5n-3 undergoes a desaturation to tetracohexaenoic acid (C24:6n-3), which generates DHA requiring an additional chain-shortening step (i.e. β-oxidation) in the peroxisome that involves multifunctional enzyme delta-4-desaturase as described by Sprecher (2000). In addition, a retroconversion of DHA into EPA takes place (Sprecher and others 1995; Willumsen and others 1996; Sprecher 2000). A similar desaturation-elongation cycle occurs in the conversion of LA to AA (Cherian and Sim 1995).

Conversion of ALA into LCPUFA in the yolk is not highly effective, and largely caused by the low activity of the desaturase enzymes (Fraeye and others 2012). This conversion could be inhibited by several other factors, including heredity, age and diet of the laying hen (Scheideler, Froning, Cuppett 1997; Fredriksson, Elwinger, Pickova 2006).

LA, and particularly ALA absorption in the hen and subsequent deposition in the yolk has been found to increase as hens age (Scheideler, Froning, Cuppett 1997). In fact, the yolk n-6:n-3 ratio fluctuates in the process of the laying period despite no change in diet. It has been hypothesized that older hens, having larger livers, produce larger yolks, which results in an

increased ability to absorb lipids and for a more effective conversion of ALA into LCPUFA (Fredriksson, Elwinger, Pickova 2006). However, contradictory evidence has shown that younger hens produced yolks with a greater level of LCPUFA (Nielsen 1998). The authors suggested that because these hens produce eggs with smaller yolks and also have lower laying frequency, the yolk contains compensatory amounts of LCPUFA. Both theories have yet to be fully elucidated.

Because of competition between n–6 and n–3 PUFA for D5DE and D6DE, an excess of LA in the diet can potentially inhibit the extent of ALA conversion to EPA and DHA (Cachaldora and others 2008b). Therefore, the activities of these enzymes can be altered by the diet (Brenner 1989). In diets high in n-3, most of D5DE is used for the n-3 pathway and thus little is available to convert DGLA into AA while in a low n-3 diet, most of D5DE is available for the conversion of DGLA to AA (Emken and others 1993). As an example, flaxseed's inherently low n-6:n-3 PUFA ratio of 0.3 ensures a lowering of the ratio from 7.5 in conventional egg yolk to approximately 2-3 in n-3 enriched egg yolk for various flaxseed diets (Botsoglou and others 1998). One study revealed that SNPs of the FADS1 (position 391 (C->A) and 468 (C->T)) in Vietnamese and European hen breeds were significantly associated with myristic acid (MYRIST), palmitic acid (PALM), palmitoleic acid (PALMO), and LA while FADS2 SNPs were associated with AA, DHA and the n-6:n-3 ratio (Khang 2006). This may also explain why MYRIST increases desaturase enzyme activity significantly (Jan and others 2004). Also, D6DE preferably receives C18 substrates for the catalytic mechanism and also allows preferential desaturation of MYRIST (Shappell and others 2001). Another cause for decreased ALA conversion is an excess of EPA and DHA in the diet. It is evident that a plateau is reached in the yolk whereby PUFA content in the sn-2 position of the PL fraction cannot exceed a certain

amount, and after this point the LCPUFA are increasingly stored in TG (Schreiner and others 2004). Addition of dietary antioxidants also seems to modulate the elongation−desaturation pathway favorably, by significantly reducing yolk AA content and increasing n-3 LCPUFA compared to hens fed flaxseed with no antioxidants. One study found that adding 100 IU vitamin E or 50 mg/kg BHT to diets containing 10% flaxseed, total n-3 PUFA increased from 4.93 mg/g yolk to 6.30 and 7.97 mg/g yolk, respectively (Hayat and others 2009).

Omega-6 Series		**Omega-3 Series**
C18:2n6		C18:3n3
LA		**ALA**
⬇	*D6DE (FADS2)*	⬇
C20:3n6		C18:4n3
GLA		**SDA**
⬇	*Elongase (ELOVL5)*	⬇
C18:3n6		C20:4n3
DGLA		ETA
⬇	*D5DE (FADS1)*	⬇
C20:4n6		C20:5n3
AA		**EPA**
⬇	*Elongase (ELOVL2)*	⬇
C22:4n6		C22:5n3
DTA		**DPA**
⬇	*Elongase (ELOVL2)*	⬇
C24:4n6		C24:5n3
TTA		TPA
⬇	*D6DE (FADS2)*	⬇
C24:5n6		C24:6n3
TPA		THA
⬇	*β-oxidation*	⬇

C22:5n6   C22:6n3

DPA   **DHA**

Figure 2.2 *Essential fatty acid conversion in humans*

Figure adapted from (Simopoulos and De Meester 2009)

2.3.3. Methods of yolk n-3 enrichment

Cruickshank (1934) was the first to report that the yolk FA composition could be modified through dietary manipulation. Decades later, the first designer egg rich in both n-3 PUFA and antioxidants was developed (Qi and Sim 1998). In Canada, a minimum of at least 300 mg n-3 PUFA in 1 egg is required for an n-3 content claim. According to the Canadian Egg Marketing Agency, n-3 enriched eggs currently account for 15% of the Canadian egg market (Flax Council of Canada 2014).

There are two ways to impart desirable n-3 PUFA into eggs. The first is through adding ALA-rich ingredients, like flax, to the hen's diet which results in a non-proportional increase in yolk LCPUFA (Grobas and others 2001; Gakhar and others 2012). After consumption, ALA can be converted into EPA and DHA by the hen's liver, and then secreted into the yolk in smaller amounts (Beynen 2004). EPA is largely non-responsive to n-3 ingredient inclusion, particularly if the source is of plant origin, with yolk levels shifting within a narrow range (refer to Table 2.5). This is because the majority of it is converted to LCPUFA such as DPA and DHA (Aymond and Van Elswyk 1995; Cachaldora and others 2008a). This effect has been demonstrated in numerous studies using marine fish or seal blubber oils. The alternative route to impart n-3 PUFA into eggs is through sources of pre-formed DHA, including fish oil or dried marine algae, which provide significant linear enrichment of LCPUFA (Gonzalez-Esquerra and

Leeson 2001; Alvarez and others 2004; Šefer and others 2011). Regardless of the dietary substrate, yolk saturation of LCPUFA is eventually achieved, as defined by a plateau in enrichment. In fact, no increase in n-3 LCPUFA was reported between eggs from hens fed 8% compared to 16% flaxseed (Cherian and Sim 1992). Furthermore, Schreiner and others (2004) found that when seal blubber oil was fed to hens in excess of 1.25% of the diet, n-3 LCPUFA in the sn-2 position of PC and PE plateaued. Feeding beyond 1.25% also caused an increase of n-3 LCPUFA in the TG, albeit a much smaller increase compared to the alteration of these fatty acids in the PL. The authors concluded that when more unsaturated fat is fed to hens beyond possible enrichment threshold, the hen has the capacity to saturate the fat because the unsaturation of yolk lipids must be within certain limits for biological and reproductive reasons.

Since the late 1990's, enriched eggs produced through flaxseed supplementation have become available on the market around the world (Surai and Sparks 2001). N-3 eggs are sold at a premium, sometimes double or triple the price of regular eggs. Eggs procured from hen's fed fish oil are even more expensive. The reason for the increased price is mainly due to the high cost of purified fish oil, and additional antioxidants to preserve the feed properly. Therefore, using vegetable-based ingredients for feed is more cost effective and easier to acquire. In addition, n-3 PUFA from vegetable sources are retained in yolk fat just as efficiently as from marine sources (Garcia-Rebollar and others 2008). Since DHA-enriched products are well-marketed, consumers may perceive these eggs as being more nutritious. Burnbrae Farms' "Omega Plus" eggs from hen's fed flax, alfalfa, corn and fish oil contain more DHA, Vitamin B12, lutein and folate than their "Omega-3" eggs from hens fed a regular flax-based diet. Currently, canola oil, fish oil and flaxseed are the only available sources for the Canadian egg industry to incorporate n-3 PUFA in the hen's diet.

As depicted in Table 2.5, it can be observed that ALA can be significantly increased in vegetable diets with limited changes in DHA, whereas fish oil diets increase DHA levels. It is also clear that DHA plateaus occur quickly, particularly in vegetable based diets. Flaxseed can be ground in order to increase lipid absorption and deposition of FA into egg yolk slightly (Scheideler and Froning 1996), however increased oxidation of the feed must be considered. Microencapsulation of sensitive lipids may offer more protection from oxidation (Kim and others 2000). Diets combining both vegetable and marine sources of n-3 PUFA are ideal in order to yield equal deposition of desirable PUFA.

Table 2.5

Alpha-linolenic acid and docosahexaenoic acid content of egg yolk from hens fed various dietary treatments

Dietary Treatment	% of total fatty acids			Reference
	ALA	EPA	DHA	
Vegetable oil				
4% sunflower oil	0.19	0.11	0.48	(Baucells and others 2000; Farrell 1998)[ac]
4% rapeseed oil	0.73	0.08	1.01	(Baucells and others 2000)[a]
1% flaxseed oil	1.39	0.15	1.44	(Rizzi and others 2009)[a]
4% flaxseed oil	4.87	0.25	1.56	(Baucells and others 2000)[a]
6% flaxseed oil	6.49	NR	1.45	(Antruejo and others 2011)[a]
6% chiaseed oil	10.98	NR	1.15	(Antruejo and others 2011)[a]
Oilseed				
25% rapeseed	1.49	NR	1.24	(Antruejo and others 2011)[a]
5% flaxseed	2.01	NR	1.83	(Scheideler and Froning 1996)[a]
10% flaxseed	4.87	0.20	1.70	(Ferrier and others 1995; Scheideler and Froning 1996)[ac]
15% flaxseed	7.07	NR	1.78	(Antruejo and others

				2011; Scheideler and Froning 1996)[ac]
20% flaxseed	10.70	0.20	1.80	(Ferrier and others 1995)[a]
7% chiaseed	2.76	NR	5.48	(Ayerza and Coates 1999)[a]
14% chiaseed	5.17	NR	3.97	(Ayerza and Coates 1999)[a]
21% chiaseed	9.04	NR	3.08	(Ayerza and Coates 1999)[a]
25% chiaseed	10.48	NR	0.93	(Antruejo and others 2011)[a]
28% chiaseed	16.52	NR	1.24	(Ayerza and Coates 1999)[a]

Fish oil

0.5% fish oil	0.28	0.11	2.07	(Van Elswyk, Dawson, Sams 1995)[b]
1.5% fish oil	0.38	0.25	3.09	(Van Elswyk, Dawson, Sams 1995[b]; Scheideler and Froning 1996[a])
2% fish oil (microencapsulated)	1.49	0.27	2.20	(Lawlor and others 2010)[b]
3% fish oil	0.36	0.43	3.73	(Van Elswyk, Dawson, Sams 1995)[b]
4% fish oil	0.44	0.92	3.18	(Baucells and others 2000)[a]
4% fish oil (microencapsulated)	1.84	0.60	2.25	(Lawlor and others 2010)[b]
5% fish oil	0.36	1.00	5.27	(Farrell 1998)[a]
6% fish oil (microencapsulated)	1.94	1.11	4.49	(Lawlor and others 2010)[b]

Combination

3% fish oil + 1% rapeseed oil	0.51	0.64	2.84	(Baucells and others 2000)[a]
3% fish oil + 1% sunflower oil	0.32	0.58	2.72	(Baucells and others 2000)[a]
3% fish oil + 1% flaxseed oil	2.26	0.58	3.80	(Farrell 1998)[a]
3% fish oil + 1% flaxseed oil	1.81	0.63	2.83	(Baucells and others 2000)[a]
2% fish oil + 2% flax	2.37	0.66	2.62	(Baucells and others 2000)[a]
2% fish oil + 1% flaxseed oil + 1% canola oil	2.32	0.45	3.38	(Farrell 1998)[a]
2% fish oil + 2% rapeseed oil	0.58	0.43	2.48	(Baucells and others

				2000)[a]
2% fish oil + 2% sunflower oil	0.31	0.39	2.30	(Baucells and others 2000)[a]
1% fish oil + 3% rapeseed oil	0.66	0.18	1.76	(Baucells and others 2000)[a]
1% fish oil + 3% sunflower oil	0.26	0.17	1.61	(Baucells and others 2000)[a]
1% fish oil + 3% flaxseed oil	3.71	0.33	1.99	(Baucells and others 2000)[a]

NR = value not reported
[a]unchanged reported values (% of fatty acids)
[b]calculated % of total fatty acids ((fatty acid / total fatty acids)*100)
[c]average of reported values

2.3.4. Health benefits of consuming n-3 enriched eggs

Egg consumption significantly declined in Western countries like Canada when restrictions on dietary cholesterol were proposed in 1972 (Jiang, Sim, Nakai 1994; Van Elswyk 1997). In fact, egg consumption in the USA dropped from a peak of 402 eggs per capita in 1945 to 232 eggs per capita in 1999 (Agriculture Marketing Research Center 2014). However, egg consumption rebounded in the late 1990's, especially with the advent of functional eggs. As shown in Table 2.4, the cholesterol content of one egg is approximately 195 mg. Although Health Canada recommendations for dietary cholesterol are to be "as low as possible while consuming a nutritionally adequate diet", Dietitians of Canada (2014) and the American Heart Association advises the general population to limit intake to less than 300 mg per day. This recommendation is mostly based on data derived from animal studies where supraphysiological doses of cholesterol were fed in order to produce atherosclerosis, and some researchers believe this recommendation should be re-evaluated (McNamara 2014). For those with elevated circulating low density lipoprotein (LDL) cholesterol (higher than 100 mg/dl) or CHD, the limit drops to 200 mg per day, still allowing one egg per day granted that other animal products are limited in the diet.   However, clinical and epidemiological studies have illustrated that dietary

cholesterol is not the major determinant of plasma cholesterol level in healthy individuals. As such, it has been suggested that there is no need to set restrictions on egg consumption, especially in healthy individuals (McNamara 1997). It was concluded that consumption of up to one egg per day is unlikely to have substantial overall impact on the risk of CVD or stroke among healthy adults based on the results of two prospective cohort studies that followed a total of 37,851 and 80,082 men and women, respectively (Hu and others 1999). However, among participants who became diabetic during the follow-up period of 8 years, daily egg consumption doubled CHD mortality. A recent meta-analysis indicated a positive dose-response association between egg consumption and risk of CVD and diabetes (Li and others 2013). Some studies suggest that consuming up to 14 conventional eggs per week have no effect on blood lipid levels (Vorster and others 1995), especially if the saturated fat level of the total diet remains low (Edington and others 1989; Garwin and others 1992). However, in persons at risk for CVD, recent findings suggest that regular consumption of egg yolk should be avoided due to significant increases in carotid plaque area (Spence and others 2012).

Consuming n-3 eggs can help to balance the overall n-6:n-3 ratio in the diet. Enriched eggs may also confer similar metabolic and cardiovascular advantages as when fish is consumed, suggesting that n-3 PUFA in eggs and fish are absorbed, distributed and metabolized in a similar manner (Riediger and others 2009; Samman and others 2009). Apart from the benefits of an increased n-3 PUFA profile, AA levels are significantly reduced in n-3 enriched eggs. Since eggs are naturally rich in AA, attenuating AA and its subsequent metabolism to eicosanoids is believed to be a targeted effect underlying some of the benefits of n-3 PUFA (Larsson and others 2004). Sindelar and others (2004) reported that consumption of just one n-3 enriched egg resulted in elevated serum levels of ALA. Yannakopoulos and others (1999) reported that plasma

total cholesterol (TC) was significantly reduced in humans aged 41–50 years who consumed n-3 eggs, while HDL cholesterol was raised. Male participants experienced enhanced n-3 levels in blood platelet PL after consuming n-3 eggs (Ferrier and others 1995).

Daily consumption of 7 enriched eggs per week among 56 healthy adults increased EPA, DHA and total n-3 PUFA, and decreased n-6:n-3 ratio in plasma (Farrell 1998). Another study reported that the majority of those who followed a low-fat, low-cholesterol diet plan could consume as many as 12 n-3 enriched eggs per week without an increase in total or LDL cholesterol (Lewis, Schalch, Scheideler 2000).

Another study involved feeding 4 n-3 eggs (using 10 or 20% ground flaxseed in the hen's diet) per day for 2 weeks in 28 healthy males with an average age of 33 (Ferrier and others 1995). Although the levels of plasma TG were inconsistent following consumption of n-3 eggs in comparison with their preliminary study (Ferrier and others 1992), there was a significant rise of DHA in platelet PL consuming n-3 enriched eggs in both studies. Accumulation of ALA and DHA, appeared to reduce platelet aggregation, thus reducing the risk of CHD. In a longer-term study, it was found that consumption of 4 enriched eggs (using 10% fish oil in diet) per day for 4 weeks did not alter mean plasma cholesterol concentration in 12 healthy adults (aged 19-49) but did significantly lower blood pressure, whereas control eggs significantly increased cholesterol concentration and had no effect on blood pressure (Oh and others 1991).   Also, mean plasma TG concentration was decreased by enriched eggs but increased by control eggs, though not always significantly different. The authors suggested that this could have been due to an insufficient amount of n-3 PUFA obtained from the enriched eggs over a short time period to cause a consistent reduction of TG, compared to direct consumption of fish oil in other published studies (Oh and others 1991).

In a similar study by Jiang and Sim (1993), plasma TC and LDL cholesterol levels were raised in subjects who consumed conventional eggs but were unchanged in those who consumed n-3 PUFA-enriched eggs. Intake of two conventional eggs per day did not affect plasma HDL cholesterol and plasma TG levels, but the intake of 2 n-3 PUFA-enriched eggs per day resulted in a significant elevation in HDL and a reduction in plasma TG. Consuming conventional eggs tended to decrease both HDL/TC and HDL/LDL ratios, whereas consuming n-3-enriched eggs tended to increase them. The n-3 PUFA contents in plasma lipids of subjects who consumed n-3 PUFA-enriched eggs were also found to be elevated.

The available literature suggests that depending on the amount consumed, n-3 eggs have a positive impact on indices of health and that enriched eggs should be considered more healthful than conventional eggs.   The increasing awareness of these benefits may have helped the egg industry rebound from declining consumption due to concerns over dietary cholesterol (Asselin 2005). However, there exists a population of 'responders' whose plasma total and LDL cholesterol is easily affected by dietary cholesterol, and egg consumption, enriched or otherwise, is not recommended (Lewis, Schalch, Scheideler 2000). Genetic differences aside, eggs can be considered a valuable source of essential nutrients for the majority of people.

2.3.5. Challenges and limitations of enrichment

A serving size of 50 g, equivalent to 1 egg, must contain a minimum of 300 mg total n-3 PUFA in order to be labeled as "n-3" (Health Canada 2014b). In addition, an ideal enriched eggs should contain a low n-6:n-3 ratio and have similar sensory attributes to conventional eggs. However, three main challenges exist when considering egg enrichment.

1) Degree of enrichment: The mechanism regulating LCPUFA enrichment is poorly understood, yet a number of factors have been considered for their low incorporation into egg yolk. These include the rate limiting effects of the desaturase and elongase enzymes (Nakamura and Nara 2004) and selective oxidation of the intermediary products of DHA from ALA, particularly EPA (Palmquist 2009). Also, selective incorporation of desirable FA into various lipid fractions confers limitations for what can realistically be achieved. Levels of ALA in eggs from hens fed a 20% algal diet were higher in the TG fraction (2.5 g/100 g total FA) than in the PL fraction (0.4 g/100 g total FA). In contrast, the LCPUFA were found in higher concentrations in the egg PL fraction than in the TG fraction (Fredriksson, Elwinger, Pickova 2006). It is unknown whether other compounds are affected by the diet. There may be potential for interactions with other dietary ingredients, which may affect the metabolism of nutrients of interest.

2) Cost and feasibility: People may not eat fish due to lack of accessibility, taste preference, allergies, or for ethical reasons. Therefore, the incorporation of n-3 PUFA in eggs is an alternative way to increase the intake of this nutrient, since eggs and egg products occur widely in people's diets, in items such as egg dishes, bakery products, mayonnaise, and salad dressing. In fact, compared to traditional eggs, the creation of enriched eggs enhances the health standard of eggs and improves their image. Unfortunately, heavy reliance on a limited number of ingredients (flax and fish) in industry for enrichment can result in problems when growing or market conditions are not ideal. This can also result in increased cost of ingredients, especially marine products. Therefore, there is a need to test novel ingredients as sources of n-3 PUFA for partial replacement in layer diets to reduce this burden. One such ingredient is hemp, which is

not currently eligible for use in animal diets in Canada due to the limited safety and efficacy data available.

      3) Sensory quality: The maximum level of *any* n-3 ingredient that can be used in the layer diet is limited not only by cost and degree of enrichment, but also due to the production of eggs with an altered sensory profile, including fishy flavour. Often when enrichment plateaus, sensory quality becomes affected as well. There may also be potential for interactions with other dietary ingredients, which may affect sensory quality. For example, rapeseed and CM has the potential to cause fishy taint in eggs (Bolton, Carter, Jones 1976; Pearson and others 1983a). Therefore, it is imperative that commercial laying hen diet formulations take sensory quality into consideration.

2.4. Sensory evaluation of n-3 enriched eggs

      Sensory analysis is a multidisciplinary science that uses human panelists and their senses of sight, smell, taste, touch and hearing to measure the sensory characteristics and acceptability of food products (Martens 1999). Sensory evaluation of any food is essential because there is no instrument that can entirely replace the human response. It can be applicable in many aspects of the food industry, including product development, product improvement, quality control, storage studies and process improvement. Sensory panels must be conducted under controlled conditions, using appropriate experimental designs, test methods and statistical analyses in order to produce reliable and reproducible data. There are two types of sensory panels used in research; untrained (consumer) and trained. Untrained panels typically utilize a hedonic scale to measure acceptability and possibly purchase behavior, however these panels require a large number of panelists, and information about specific attributes that are detected cannot be

gathered. On the other hand, trained panels gather a large amount of information about specific attributes while only requiring a total of 8-12 panelists.

It is important that functional foods maintain similar sensory characteristics compared to regular foods in order to remain palatable to consumers. Otherwise, the likelihood of consumption is drastically decreased. This is most problematic when functional foods are developed for the sole purpose of reducing the risk for disease development and progression. A potential downside of egg enrichment is the possibility of a sensory profile that is different, or more concerning, worsened, compared to its conventional counterpart. Since the egg shell is porous, allowing for gaseous exchange in and out of the egg, migration of volatile compounds from packaging materials or the surrounding environment through the shell can result in altered egg aroma and flavour (Matiella and Hsieh 1991). However, altering the hen's diet can also have a profound effect on egg sensory quality. Typically in the case of n-3 enriched eggs, the greatest sensory concern lies in the potential for off-aromas and off-flavours, usually described as 'fishy'. It was once believed that strongly flavoured feeds resulted in strongly flavoured eggs, however it is now understood that this is a much more complex issue.

2.4.1. Quality of dietary ingredients

Feeding fish oil to hens has often resulted in fishy taint in eggs. It was once believed that processing these oils using a method known as deodourization to remove ketones and benzene-containing compounds would solve this problem. However, results from Gonzalez-Esquerra and Leeson (2000) showed that deodourized fish oil in the layer diet was no different than regular fish oil in terms of aftertaste or off-flavours in the eggs. After this was demonstrated, this hypothesis was dismissed and the majority of subsequent research has focused more on oxidation of feed components and specific dietary composition.

2.4.2. Feed oxidation protection

An increase in the content of n-3 PUFA in the egg may increase its susceptibility to lipid oxidation, which some researchers believe to result in off-flavour and off-aroma (Van Elswyk, Dawson, Sams 1995; Cherian, Wolfe, Sim 1996). Fishiness is thought to be more of a problem during egg processing (Woods and Fearon 2009).

To attempt to inhibit feed oxidation, which ultimately may lead to impaired sensory quality through the deposition of oxidized lipids into the egg (Galobart and others 2001; Hayat and others 2010b), several methods have been employed with varying degrees of success. Most commonly used has been the supplementation of antioxidants into an enriched diet, including mixed tocopherols which has resulted in a significant improvement in oxidative stability of eggs, compared with those without tocopherols (Cherian, Wolfe, Sim 1996).

In fact, Cherian, Wolfe and Sim (1996) and Galobart and colleagues (2001) found that the inclusion of tocopherols (200 mg/kg) resulted in a significant reduction in thiobarbituric acid reactive substances (TBARS) in enriched eggs. Unfortunately, in other studies, there was no significant improvement in lipid stability and/or sensorial characteristics resulting from antioxidant supplementation (Leeson, Caston, MacLaurin 1998; Hayat and others 2010a; Hayat and others 2010b). In fact, contrary results have showed that lower levels of Vitamin E may be better at preventing these off-flavours (ie. 10 vs. 100 IU Vitamin E) (Leeson, Caston, MacLaurin 1998). Also, several authors have demonstrated that Vitamin E over 120 ppm in conventional diets can act as a pro-oxidant in egg yolk, resulting in an elevation in TBARS (Chen and others 1998; Gebert and others 1998; Franchini and others 2002). Large doses of Vitamin E should be used sparingly in general, but research suggests that this tactic may be helpful when antioxidants are paired with sufficient amounts of unstable lipids, like n-3 PUFA. This effect should be

investigated with other antioxidants, as this pro-oxidant effect is likely to occur. Furthermore, protecting n-3 oils through microencapsulation may prove to be an efficient method of preserving FA and protecting them from oxidation which would result in decreased sensory quality (Lawlor and others 2010). Regardless of the level of antioxidant inclusion in the diet, off aromas and flavours still occur most likely due to the egg's FA composition with increased n-3 levels.

2.4.3. Dietary composition

Contrary to those who believe that off-aromas and flavours in enriched eggs are the direct result of lipid oxidation, other researchers have proposed that the culprit is more likely to be a combination of lipid and non-lipid substances in the hen diet. These components can affect the concentration of volatile compounds, presence of trimethylamine (TMA) in addition to lipid oxidation products within the egg (Jiang and others 1992; Van Elswyk, Sams, Hargis 1992; Van Elswyk, Dawson, Sams 1995; Scheideler, Froning, Cuppett 1997; Leskanich and Noble 1997; Parpinello and others 2006).

The laying hen diet that is chosen to yield an enriched egg is ultimately dependent on the desired FA profile of the egg yolk. If the more healthful LCPUFA such as DHA are desired, then fish or algal ingredients are typically used in industry to achieve this outcome. This approach is more expensive and the fatty acids are highly sensitive to oxidation due to the increased number of double bonds, however smaller quantities are typically needed in the diet (Aymond and Van Elswyk 1995). If oxidation does occur, these fatty acids and their oxidized products are not only undesirable but are also known to cause adverse biological effects in humans (Aymond and Van Elswyk 1995).

An immediate concern with supplementing a hen's diet with fish oil or fish meal is the negative effects on organoleptic quality as eggs procured from these hens have been found to possess a pronounced fishy aroma and flavour, depending on the dietary concentration and the particular type of fish used (Surai and Sparks 2001). Aro and others (2011) found that the use of 5% fish oil in hen diets yielded eggs with significantly lower sensory scores (higher off-aroma and flavour) but were still considered generally acceptable compared to the control eggs. Ceylan and others (2011) had similar findings, but these authors used a lower level of fish oil inclusion at 3%. Hammershøj (1995) found that taste and general sensory impression were of lower grades for eggs procured from hens consuming diets with 1.5 and 3% fish oil compared with animal fat diets. Differences in source of fish oil or fish meal were also observed, likely in part due to differences in their fatty acid profile. For example, the addition of sardine oil did not influence egg flavour (Vondell and Putnam 1945), but menhaden fish oil did (Holdas and May 1966). Diets containing British Columbia herring meal had higher flavour quality than the use of hake meal and Canadian Atlantic herring meal (Koehler and Bearse 1975). Peruvian anchovy oil at a low inclusion level of 1.5% produced off-flavoured eggs (Koehler and Bearse 1975). Menhaden fish oil has been investigated extensively for its use in layer diets and has been found to be generally acceptable at levels less than 1.5% and 8% for oil and meal, respectively (Van Elswyk, Sams, Hargis 1992; Marshall, Sams, Van Elswyk 1994; Van Elswyk, Dawson, Sams 1995; Scheideler, Froning, Cuppett 1997; Van Elswyk 1997; Gonzalez-Esquerra and Leeson 2000). Other types of fish, such as Cantabrian blue fish oil at 1.5% have also been found acceptable (Laca, Paredes, Díaz 2009). In another study, researchers found that only 1 out of 8 trained panelists were able to distinguish between eggs from hens fed 1.25% seal blubber oil or conventional eggs (Schreiner and others 2004). As the level of inclusion rose, so did the

panelists ability to pick out the treatment eggs. However, because a plateau in n-3 deposition was observed beyond 1.25%, it would not be worthwhile to feed greater amounts, and in turn affect sensory quality.

On the other hand, protecting these delicate oils from oxidation has been successful, as demonstrated by Lawlor and others (2010) who showed that as much as 6% microencapsulated fish oil (MFO) in the layer diet were acceptable when eggs were scrambled. However, when these same eggs were hard-boiled, off-flavour and sulphur flavour was increased significantly compared to the 4% MFO group, which was not different from the control group. Therefore, commonly used cooking practices should be taken into consideration when designing diets to ensure that deleterious flavours or aromas do not occur.

Despite the fact that greater increases in egg DHA (3.5%) and EPA (0.52%) have been achieved using lower amounts of fish oil than with greater numbers of oleaginous seeds (Hargis and Van Elswyk 1993), there are a number of advantages in using vegetarian feed. The idea of using flaxseed for n-3 egg production began when sensory studies reported strong "fishy flavours" from fish oil-based n-3-enriched eggs (Leskanich and Noble 1997). Flaxseed has been a popular choice as a vegetable source of n-3 PUFA for animals but there have been reports that eggs from hens offered flaxseed also have a fishy aroma or flavour, similar to that found in eggs from hens offered fish products. In such terrestrial diets, the plant oils contain a substantial amount of ALA, which deposit into the egg yolk, along with a small proportion of longer chain n-3 PUFA such as DHA, which the hen liver converts to this form. The benefit of such diets is that they are comparatively inexpensive and less prone to oxidation than the former method. Many would argue, however, that important long-chain n-3 PUFA such as DHA that would be deposited in this case would not be as abundant in eggs from fish fed hens. However, if the

threshold for fish oil in hen diets is to be respected to avoid sensory impairment (ie. 1.5% for fish

oil), yolk DHA deposition is likely to not exceed 138 mg/egg (Herber and Van Elswyk 1996),

which can already be achieved using vegetarian ingredients (ie. flaxseed). In fact, eggs were

acceptable to consumers with as much as 5% FO in the layer diet (Cloughley and others 1997).

However, another study found that the taste of Madeira cakes made with eggs from hens fed 2%

FO were worse, as compared to those cakes which included eggs from hens fed palm butter,

grape seed oil, or lard (control) at the same level of inclusion (Tallarico and others 2002). In a

study which incorporated 10% flax seed in the layer diet, it was determined that the resultant

eggs were acceptable to consumer panelists, however a trained panel was able to detect

differences in flavour, aroma, off-flavour and overall difference (Hayat and others 2010a). In

another study using 10% *ground* flaxseed, sensory acceptability was also maintained (Mridula

and others 2012). Levels of flaxseed greater than 10% however have showed lower acceptability

along with 'fishy' and off-flavours compared to conventional eggs (Caston, Squires, Leeson

1994; Scheideler, Froning, Cuppett 1997; Leeson, Caston, MacLaurin 1998). Moreover,

Scheideler, Froning and Cuppett (1997) found that appearance and flavour scores were slightly

lower for 10 to 15% ground flaxseed compared to the same level in the form of whole seed. This

could be attributed to either enhanced absorption of FA in the hen, or oxidation occurring in the

feed before it is consumed.

Overall, the conservative approach to prevent fishy taint is to include no more than 5%

flaxseed, or 1.5% fish oil in layer diets (Scheideler, Froning, Cuppett 1997; Surai and Sparks

2001; Woods and Fearon 2009). However, the sensory outcome is highly dependent on the level

of inclusion, type (oil/seed), processing (whole/ground) and mixture with other ALA ingredients.

For example, Ayerza and Coates (2002) found that combining flaxseed with chiaseed in the hens'

diet, but keeping the flaxseed content below 5% of the diet, successfully increased yolk ALA without any adverse effect on egg flavour or aroma (Woods and Fearon 2009).

However, other promising ingredients rich in desirable FA may be used as an alternative to fish and flax. For example, marine algae has been found to be a promising ingredient to enrich DHA in eggs, without compromising the sensory profile (Herber-McNeill and Van Elswyk 1998). In fact, the latter authors found that the inclusion of 4.8% of a marine micro algal product was as acceptable as control eggs in terms of egg flavour. Along with 2% sardine oil, 10% of each of the following marine algae were included in the laying ration; *M. pyrifera, S. sinicola* and *Enteromorpha spp* (Carrillo and others 2008). The authors found no flavour differences, but warned that using algae inclusion levels above 10% should be avoided as this could affect the acceptance of the feed, lower the egg production or cause diarrhea due to the high mineral content in the seaweed.

The inclusion of Chia (*Salvia hispanica L.*) in hen diets has been found to produce similar sensory characteristics as conventional eggs and even at maximum inclusion levels (30%), it did not change the aroma or flavour profile of cooked eggs. This is especially important because chiaseed contains more n-3 PUFA than flaxseed. Moreover, chia-fed hens produced eggs with a lower score and hence a milder flavour compared to the control (Ayerza and Coates 1999). This finding is similar to results from Rokka and others (2002), who found that feeding 5% camelina seed oil resulted in an improved sensory profile compared to a control diet. More interesting is that the sensory profile was improved compared to a flax seed oil diet, suggesting it may be a good alternative to flax, at least from a sensory point of view (Rokka and others 2002). Dietary levels up to 10% of extruded camelina meal was deemed acceptable, however two panelists noted some fishiness (Kakani and others 2012).

Egg sensory characteristics are complex. It has been demonstrated that not all breeds or chickens within a breed produce off-flavoured eggs, and even feeds without fish meal can produce similar negative sensory effects, and that the level of ingredient inclusion into the diet has an effect on the resulting eggs.

2.4.4. Colour

The intensity of yolk colour is due to the presence of yellow and red oxycarotenoids, which are xanthophyll pigments found in some plants (Karunajeewa and others 1984; Palmer & Kempster 1919). There are 2 main classes of naturally occurring carotenoids: (1) carotenes (β-carotene, α-carotene), which are hydrocarbons, are either linear or cyclized at one or both ends of the molecule, and (2) xanthophylls, the oxygenated derivatives of carotenes (Sajilata, Singhal, Kamat 2008). Egg yolks range in colour from pale yellow to deep orange depending on the concentration of these natural pigments. For example, a typical Canadian wheat/barley-based diet will result in a pale yellow yolk, while a European corn/lucerne/alfalfa-based diet yields a darker yellow yolk. In addition to such ingredients, pigments to impart richer yolk colours can be naturally obtained from marigold, tomato or paprika (Fletcher and Halloran 1983; Nys 2000) or synthesized artificially, although this is seen as negative practice in industry.

Although macronutrients remain the same regardless of yolk colour, there may be small changes in some of the micronutrients. Hydroxyl compounds (Singh, Pathak, Akhilesh 2012) like xanthophylls are normally absorbed from the feed in the following order; lutein, luteinmono- and diester, 3'-oxolutein and zeaxanthin. These carotenoids not only provide colour, but can also exhibit antioxidant function contributing to eye health (Carpentier, Knaus, Suh 2009). Lutein in particular has demonstrated biological activities that have attracted great attention in relation to human health. In particular, high lutein intakes have been associated with a lower risk

for developing CVD, several types of cancer, cataracts and age-related maculopathy (Granado, Olmedilla, Blanco 2003). Both lutein and zeaxanthin have been found to be helpful in the prevention of age-related macular degeneration, a leading cause of blindness in the elderly, and have been associated with lower risk of cataract extraction (Moeller, Jacques, Blumberg 2000). Carotenoid consumption through egg yolk confers the additional benefit of being a highly bioavailable source of antioxidants, since these lipid-soluble compounds are taken up by diffusion of micelles and transported to the liver in blood (Handelman and others 1999). In fact, results from an intervention study showed that after 10 days of ingesting 6 mg lutein per day the absolute serum lutein concentration was significantly higher from egg yolk (528.5±56.3 nmol/L) compared to lutein supplements (288.1±31.5 nmol/L) or spinach (326.1±43.5 nmol/L) (Chung and others 2004).

Some carotenoids, like beta-carotene, have nutritional value as a precursor to Vitamin A, which is essential for healthy growth and reproduction (Chytil and Ong 1976; Zile and Cullum 1983). However, dark coloured egg yolks only indicate the presence of total carotenoids, not necessarily the presence of beta-carotene alone. In fact, beta-carotene content in the yolk reaches saturation at 1%, regardless of the beta-carotene content of the hen's feed (Herber-McNeill and Van Elswyk 1998).

Although the colour of the yolk does not indicate egg quality, freshness, or nutritional value, perception of quality may lead to preferences for certain yolk colour. Conflicting results in consumer tests suggest that preferences for yolk colour may be region or individual specific, rather than a universally accepted quality parameter. In other words, in regions where hens are typically fed a corn-based diet, people tend to prefer golden yolks, whereas in areas where a wheat-based diet is more common, a pale yolk is preferred. For instance, panelists have scored

conventional eggs with a pale yolk lower for acceptability compared to more pigmented enriched eggs strictly based on appearance (Farrell 1998). Conversely, Ofosu and others (2010) found that panelists had a higher preference for egg yolks lighter in colour. Preferences among enriched eggs have also been observed. Scheideler, Froning and Cuppett (1997) found that panelists scored eggs from hens fed golden flaxseed, better for appearance compared to eggs from hens fed brown flaxseed, yielding a darker yolk. In addition, the use of pigmentation can be used as a marketing tool to help consumers identify enriched eggs from typical eggs, since enriched eggs are typically fortified with lutein as well. In many circumstances, colour changes in the yolk are statistically significant when certain instruments are used, however the perception to the human eye may be relatively insignificant, and therefore these results cannot infer what consumer preferences may be. In fact, in the majority of the aforementioned studies, sensory panelists could not differ between treatments based on colour during training sessions where red lighting was not utilized.

2.4.5. Functionality

Polyunsaturated FA are more susceptible to oxidative breakdown during cooking than MUFA or SAT leading to a greater quantity of volatile products formed (Dinh 2008). Phospholipids contain higher proportions of unsaturated FA, particularly AA (20:4), which undergoes oxidation more readily than SFA, making them an important source of volatiles during cooking (Mottram 1998). Despite this, the cooking characteristics of enriched eggs are the same as conventional eggs, and the fatty acid profile is not affected during cooking or storage of eggs for 7 weeks at room temperature (25ºC) (Van Elswyk, Sams, Hargis 1992). However, there may be some changes in relation to foaming properties. No differences have been observed in

the yolk emulsification capacity, hardiness and springiness of sponge cakes containing enriched eggs (Van Elswyk, Sams, Hargis 1992; Leskanich and Noble 1997; Singh, Pathak, Akhilesh 2012). Aro and others (2011) tested the foaming, emulsifying and gelling properties of fresh and stored hen eggs fed with a diet supplemented with 5% inclusion of oils from flaxseed, rapeseed or fish. They found that compared to control eggs, FO feed supplementation had statistically significant influences on the foaming properties of the fresh eggs. Eggs stored for 21 days lost part of their foaming properties in the fish oil group, but the foaming properties in all test groups were deemed acceptable. Also, the emulsifying properties of eggs in FO and fish oil groups were statistically different compared to the control. Only small variations were found in terms of emulsion activity, emulsion stability, and gel-forming capacity between the fresh eggs from the control group and the test groups. These results agree with Rokka and others (2002), who found that modifying the layer diet with 5% camelina seed oil did not affect the foaming, emulsifying and gelling properties of the eggs. Thus, bakeries and the egg processing industry could use this level of inclusion (5%) of various n-3 oils in layer diets without fear of affecting the egg functionality.

In boiled eggs, FO and fish oil supplementation induced off flavours in eggs, but no changes between the control group and test groups were found in the sensory properties of mayonnaise preparations (Aro and others 2011). It is likely that these off-notes become diluted when these eggs are incorporated as part of a dish. This research indicates that tainted eggs could be used in industry to create enriched food products, wherein fishiness is masked by other flavours.

2.5. New approaches to n-3-enriched eggs

2.5.1. Novel Ingredients

A number of novel ingredients that are rich in n-3 PUFA have yet to be tested for safety and efficacy in laying hen diets. The main novel ingredient of interest in the current research is hemp, specifically HS and its oil fraction. Cultivation of industrial hemp (*Cannabis sativa* L.) is permitted in about thirty countries worldwide, primarily as a source of fibre for paper, textile or composite board production (Pickering and others 2007). It was prohibited to cultivate hemp in Canada since 1938 due to concerns over the presence of the psychoactive drug component tetrahydrocannabinol (THC). However, successful breeding through the years has resulted in the development of a low THC form of industrial hemp that was legalized in Canada in 1998 (Oomah and others 2002). Currently, the Canadian Food Inspection Agency (CFIA) will not allow such ingredients for use in hen diets. Hempseed meal, which is rich in protein, has been used in hen diets in controlled experiments (Halle and Schöne 2013; Silversides and Lefrançois 2005) and it was found that measures of hen health and egg production were not significantly affected by the diets. Additionally, eggs contained lower levels of PALM, and higher levels of essential FA. The seed or oil fractions of hemp have not yet been tested as an alternative or in combination with other n-3 ingredients in hen diets to produce n-3 enriched eggs.

2.5.2. Designer Oil Blends

The strategy of the Canadian egg industry to produce n-3 enriched eggs is to either incorporate 10-20% flaxseed in the diet, or flaxseed plus a small amount of fish oil. Other companies outside of Canada have used algae-based feed to deliver more LCPUFA in the egg. Many dietary interventions have been conducted to determine the efficacy of specific ingredients to act as a source of these desirable lipids. However, it is not yet known what the optimal proportions of FA to produce eggs with the greatest amount of targeted lipids. Therefore, it is

important to determine the optimal balance of dietary FA to enhance yolk ALA conversion to DHA in order to benefit human health. The three major families of PUFA, n-3, n-6 and n-9, are all metabolized using the same group of enzymes (Le and others 2009), and desaturase enzymes display differential activity in the above order of preference (Brenner and Peluffo 1966). With this knowledge of essential fatty acid metabolism, proportions of these PUFA can be adjusted through a variety of diets, including a designer blend of vegetable oils including coconut, canola, soybean, sunflower and corn oils. This tactic would be less expensive than the current practice of using fish oil in layer diets and would also provide an option for vegetarian consumers who would not accept fish oil as a feed ingredient.

2.5.3. Dietary Interactions

There is a possibility for dietary ingredients to react negatively with one another, resulting in a sensory profile that is worsened. CM is an economical crop grown abundantly in Canada. It is a popular protein source used in animal production because of its good amino acid profile. However, CM contain precursors to TMA, a compound partially responsible for 'fishy' aroma in eggs. In hens that have a particular inherited genetic defect, consumption of CM could translate into disrupted capacity of flavin-containing monooxygenase isoform 3, FMO3, to oxidise TMA into the odourless TMA oxide (TMAO), thus accumulating in egg yolk. It is our hypothesis that this could produce an additive effect when combined with n-3 ingredients, since both ingredients used in isolation have resulted in impaired sensory quality.

CHAPTER 3: HYPOTHESIS AND OBJECTIVES

The project hypothesis, aim and objectives are as follows:

Hypothesis: Altering the lipid profile of the laying hen diet will result in a significant impact in yolk fatty acid profile, while negatively impacting the aroma and flavour of cooked eggs.

Aim: To gain a clearer understanding of the potential impact various layer diets have on resulting eggs. This information can be used in the poultry industry to optimize egg fatty acid composition for human health, while balancing the effect on sensory quality in order to maintain consumer acceptance.

Objectives: The general objective of this research was to evaluate various dietary interventions in laying hen diets in terms of egg fatty acid profile and sensory characteristics. The specific objectives were to:

1. Determine the degree of fatty acid deposition in eggs procured from hens fed maximum inclusion levels of HO and HS.

2. Enhance ALA to LCPUFA conversion in the egg yolk through designer vegetable oil blends.

3. Examine the potential for interactions between CM and n-3 PUFA leading to possible impaired sensory quality, beyond which would be possible if using these ingredients in isolation.

4. Determine the association between fatty acids and sensory characteristics.

CHAPTER 4: FATTY ACID PROFILE AND SENSORY CHARACTERISTICS OF TABLE EGGS FROM LAYING HENS FED HEMPSEED AND HEMPSEED OIL

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4.1. Abstract

Hempseed is rich in n-3 PUFA, with approximately 17% of total FA as ALA. As such, HS and its oil may be used in hen diet formulations to produce eggs enriched in essential FA. Because n-3 eggs have the potential for unpleasant aromas and flavours, the current study was designed to assess the fatty acid profile and sensory attributes of eggs procured from hens consuming diets containing HO or HS. A total of 48 individually caged White Bovan hens received 1 of 6 diets containing 4, 8, 12% HO, 10, 20% HS or 0% hemp (w/w) for 12 weeks. Total n-3 PUFA content was highest in the 12% HO group (15.3 mg/g of yolk) compared to the control (2.4 mg/g of yolk). Trained panelists (n=8) found no significant differences (P≥0.05) in aroma or flavour between cooked eggs from different dietary treatments, with the exception of sweet flavour. The 4% HO group yielded the least sweet eggs compared to the 20% HS group, which was highest. For yolk colour, L*, a* and b* values (mean±SEM) for control eggs were 61.2±0.10, 1.1±0.05, and 43.0±0.22, respectively. Addition of hemp led to significant (P<0.001) reductions in L*, and significant increases in a* and b*, with the largest changes observed in the 20% HS treatment (L*=58.7±0.10; a*=5.8±0.05; b*=60.5±0.22). The results show that hemp use in hen diets leads to increased n-3 PUFA content and colour intensity of egg yolks, but does not have adverse effects on the sensory profiles of the cooked eggs.

4.2. Introduction

Linoleic acid (LA) and ALA are essential FA that must be obtained through the diet. The latter is the parent compound of the n-3 series of FA that can be further converted into LCPUFA, like EPA, DPA and DHA once consumed. It is well established that n-3 PUFA, EPA and DHA in particular, are important for normal growth and development, thereby contributing to the overall health status of individuals (Simopoulos 1991). However, isotopic tracer studies have shown conversion from ALA into DHA in humans to be almost negligible, as reviewed by Brenna and others (2009). Current global n-3 PUFA recommendations for healthy adults are between 1-2% of total energy, however most North Americans fall short (World Health Organization 2003). Therefore, increasing n-3 PUFA consumption is important, and one way this can be achieved is through the manipulation of the fatty acid composition of regularly consumed foods that make a significant contribution to fat intake (Rymer and Givens, 2005).

Hen eggs could serve this function for several reasons. Much like humans, the hen can also convert a proportion of dietary ALA to LCPUFA that can be deposited in the egg yolk, thereby being considered a good source of DHA for lacto-ovo-vegetarians. Additionally, eggs are nutritionally dense, relatively simple and inexpensive to produce, and highly palatable to a wide range of people (Surai and Sparks 2001). To minimize exposure to mercury, certain health agencies recommend limits on fish consumption (Health Canada 2012a). Therefore, n-3 enriched eggs can be considered a safer alternative to fish and other marine sources (Farrell 1998).

In general, two approaches have been used to increase n-3 PUFA concentrations in table eggs. The first approach involves the use of fish oils, fish meals, and algal products. Because these products do not contain ALA, this approach results in a significant amount of LCPUFA in

the egg yolk, with low levels of ALA. The second approach, which is less expensive, involves

the addition of ALA-rich ingredients such as flaxseed to the hen diet. With this approach, a

proportion of dietary ALA is readily deposited into the yolk, in addition to a smaller amount that

is converted into LCPUFA by the hen's liver (Beynen 2004). There are numerous studies

published with flaxseed-feeding to hens (Novak and Scheideler 2001; Bean and Leeson 2003;

Beynen 2004; Hayat and others 2009; Ansenberger and others 2010) with positive yolk fatty acid

profiles. However, another promising vegetable source that has not been approved as a poultry

feed ingredient by certain government regulatory agencies, including those in Canada, is HS

(*Cannabis sativa* L.) and its by-products.

Hempseed contains approximately 30% oil by weight, and this oil is rich in ALA at

approximately 17% of total FA (Leizer and others 2000; Callaway 2004). Hempseed oil also

contains approximately 3-4% GLA, an n-6 PUFA that serves as an intermediate for the

formation of LCPUFA, anti-inflammatory eicosanoids and SDA, which may have similar

biological properties as EPA (Fan and Chapkin 1998; Leizer and others 2000; Whelan 2009).

Limited studies have been conducted using hemp products in laying hen feed (Cruickshank

1934; Silversides and Le Francois 2005), consequently, more evidence of the safety and efficacy

of the use of hemp in poultry diets is required prior to this crop being fully exploited as a

potential source of n-3 PUFA in animal diets.

In addition to questions of safety and efficacy, the use of novel feed ingredients in laying

hen diets must be weighed against any potential challenges to the sensory attributes of the

resultant eggs. Studies involving hen diet manipulation often use trained panelists to determine

the sensory quality of the resulting eggs, in terms of their aroma, flavour, appearance and texture.

The use of n-3 PUFA rich ingredients in the hen diet may result in the development of off-

flavoured eggs therefore sensory evaluation is an essential component to determine any potential sensory attributes not normally present in conventional eggs when a source of n-3 PUFA is used. Therefore, the objective of this study was to assess the impact of including HS and HO in laying hen diets on the deposition of n-3 PUFA into the resultant eggs, and the assessment as to the potential for changes in the sensory attributes of the resultant eggs.

4.3. Materials and Methods

4.3.1. Bird housing and environment

Forty-eight White Bovan laying hens at 19 weeks of age were used in this experiment. Hens were caged individually (1032 cm² floor space allowance), under semi-controlled environmental conditions (temperature was controlled via supplemental heat and/or ventilation) including exposure to a 16-hour photoperiod. Feed and water were provided for *ad libitum* consumption. Individual cage feeders were fitted with weighted screens that, when placed on top of feed, minimized feed wastage. All of the procedures used during this study were approved by The University of Manitoba's Animal Care Protocol Review Committee (see Appendix A), in accordance with recommendations established by the Canadian Council on Animal Care (Canadian Council on Animal Care 1993).

4.3.2. Diets

The diets used in this study are shown in Table 4.1. Diets were isoenergetic and isonitrogenous, and formulated to meet the requirements of laying hens consuming 100 grams of feed per day (National Research Council 1994). Diets were prepared by including 4, 8 or 12%

HO, or 10 or 20% HS (w/w) (Hemp Oil Canada, Ste. Agathe, MB, Canada). The control diet

contained neither HS nor HO.

Table 4.1: Ingredient composition of layer diets used to determine the impact of dietary hempseed oil and hempseed inclusion on egg sensory quality, yolk lipids and yolk colour

Ingredients (%)	0%H	4%HO	8%HO	12%HO	10%HS	20%HS
Ground soybean meal	29.59	29.59	29.59	29.59	24.56	19.64
Ground wheat	28.85	28.85	28.85	28.85	27.18	25.00
Ground barley	15.00	15.00	15.00	15.00	15.00	15.00
Corn Oil	12.00	8.00	4.00	0.00	8.81	5.87
HS	0.00	0.00	0.00	0.00	10.00	20.00
HO	0.00	4.00	8.00	12.00	0.00	0.00
Limestone	10.15	10.15	10.15	10.15	10.03	10.05
Vitamin Mineral Premix	2.50	2.50	2.50	2.50	2.50	2.50
Dicalcium Phosphate	1.57	1.57	1.57	1.57	1.54	1.50
Salt	0.33	0.33	0.33	0.33	0.34	0.34
DL-Methionine	0.01	0.01	0.01	0.01	0.00	0.00
L-Lysine-HCl	0.00	0.00	0.00	0.00	0.04	0.10
Calculated Composition						
AMEn (Poultry; Kcal/kg)	2998.00	2998.00	2998.00	2998.00	2988.00	2988.00
Crude Fat (%)	13.23	13.23	13.23	13.23	13.00	13.00
LA (%)	6.96	7.06	7.16	7.26	6.91	6.99
ALA (%)	0.17	0.80	1.43	2.07	0.65	1.13
Ratio LA:ALA	40.94	8.83	5.01	3.51	10.63	6.19
Crude Protein (%)	19.00	19.00	19.00	19.00	19.00	19.00
Total Lysine (%)	0.96	0.96	0.96	0.96	0.95	0.95
Calcium (%)	4.10	4.10	4.10	4.10	4.05	4.05
Total Phosphorus (%)	0.67	0.67	0.67	0.67	0.74	0.81
Available Phosphorus (%)	0.45	0.45	0.45	0.45	0.45	0.45
Sodium (%)	0.15	0.15	0.15	0.15	0.15	0.16
Chloride (%)	0.25	0.25	0.25	0.25	0.25	0.25
Methionine (%)	0.30	0.30	0.30	0.30	0.31	0.32
Threonine (%)	0.72	0.72	0.72	0.72	0.72	0.72

H= Hemp; HO= Hempseed oil; HS= Hempseed; AMEn= Nitrogen corrected apparent metabolisable energy; LA= Linoleic acid; ALA= Alpha-linolenic acid

Our hemp ingredients were delivered with a supplier certificate analysis indicating a maximum allowable limit of 0.000010% THC content in the HO and HS. Diets were predominantly a wheat-soybean meal-barley mix, using limestone as the calcium source. Crude fat was kept constant across treatments at about 13%, a level determined based on the highest inclusion rate of the intact HS (20%). To maintain the same crude fat content across treatment diets, corn oil was chosen as the additional fat source due to its low ALA content, therefore any effect the diets had on yolk FA could be attributed to the presence of HO or HS. Because of the high fat content of the diets, additional alpha-tocopherol (vitamin E) was added to all diets at 150 international units (IU) per kg of feed as part of the vitamin-mineral premix. The ratio of LA to ALA varied across treatment diets, with the largest difference seen in the control at 40.94, and 3.51 in the 12% HO diet.

4.3.3. Dietary analysis

Feed samples were analyzed for dry matter, crude protein and crude fat according to established procedures (AOAC International 1995). The fatty acid composition of the test diets was determined using standard gas chromatographic techniques of the fatty acid methyl esters (AOAC International 1990), using C17:1 methyl ester as an internal standard. Refer to Table 4.1 for diet formulations and calculated composition.

4.3.4. Experimental protocol

Hens were weighed and caged individually in roll-out style cages, and allowed an adaptation period of 10 days before being randomly assigned to receive 1 of the 6 dietary treatments (n=8 per treatment) for 12 weeks. Body weights of hens were recorded at the start of the trial, and at weekly intervals. Feed consumption was determined for the entire week and

average daily feed intake and feed efficiency was calculated. Eggs were hand collected from

each cage, egg production was recorded daily and an average egg production rate was calculated.

Prior to sensory analysis, all eggs were stored in trays in the dark at 4ºC overnight.

Figure 4.1: Picture of individual cage system with feeders
Photograph by © Erin Goldberg

4.3.5. Fatty acid analysis

Eggs from hens at 27 weeks of age (Week 8 of the experiment) were chosen for fatty acid

analysis.   Egg yolks were separated from the albumen and kept in plastic bags at -80°C until

analysis. Fatty acids were extracted from the egg yolk according to the methods of Folch, Lees

and Stanley (1957). The fatty acid composition of the yolks was determined in the same manner

as the feed (Refer to Section 4.3.3.).

4.3.6. Sample preparation

Four randomly chosen eggs from hens at 29 weeks of age (Week 10 of the experiment) of similar weight (56 ± 2g) from each treatment (collected one day before panel and kept at 4°C) were pooled and mixed for 8 seconds using a food processor (Cuisinart Little Pro Plus, Model LPPC, East Windsor, NJ, U.S.A.) and poured into 9 red glass jars with the following specifications: volume 80 ml, height 6.5 cm, internal diameter (top) 5 cm, internal diameter (bottom) 4 cm (Tradition Inc., Montreal, QC, Canada) (15 ± 0.05g per jar). The jars were then covered immediately with an aluminum weighing dish (Fisherbrand Cat No. 08-732). Jars were placed on a metal rack in a stainless steel cooking pot (10 L.) filled with 2L warm water. There were 9 glass jars containing eggs from the same treatment in every pot. The stove-top (Fridgidare Professional Series, ElectroLux Home Products, Augusta, GA, U.S.A.) was turned on high until the water began to boil (7 min), at which time the temperature was turned down to maintain a light boil (90°C) for the remainder of the cooking. The texture and temperature of the sample was checked with a digital thermometer (Fisher Scientific, Type K), and was considered cooked when a hole remained in the sample after being punctured in the center, and the internal temperature had reached at least 76ºC. The cooking process took approximately 15±1 min. No cooking oil or salt was used. All prepared samples were analyzed within 10 min after preparation was complete. Jars were kept at a constant temperature of 55°C in a heated water bath until panelists evaluated the samples.

4.3.7. Recruitment

Eight panelists (6 females, 2 males, aged between 18 and 40) were recruited (See Appendices G-I) to participate in the present sensory evaluation. The sensory analysis component of the research received ethical approval from The University of Manitoba's research

ethics board (See Appendices C-F). The panelists were students and staff of the University of

Manitoba. The criteria for participation included open availability, an interest in the panel, and

no aversion or allergies to eggs and any ingredients present in any other products to be used for

training purposes. Panelists completed a questionnaire (See Appendix K) to ensure that no

allergies were present, and written consent (See Appendix J) was obtained.

4.3.8. Descriptive analysis

Prior to analysis all panelists participated in 6 training sessions of 45 min in duration at

which time egg samples procured from experimental hens were presented coded with a randomly

selected 3-digit number. During each training session, panelists evaluated the aroma and flavour

(See Appendices L-N) of each of the randomized samples and developed an agreed vocabulary

of attributes. The final agreed descriptive vocabulary consisted of six aroma and seven flavour

attributes (Table 4.2). Samples of cooked commercial eggs, saline solution, sucrose solution,

citric acid solution, milk, cream, butter, and yogurt were also evaluated and used as reference

points for each attribute.

Table 4.2: Aroma and flavour definitions and standard products used in sensory training sessions
for cooked eggs procured from hens consuming hempseed oil or hempseed

Attribute	Definition	Standard Product[1]/Amount
Aroma		
Egg	Aroma associated with scrambled egg	blended commercial egg cooked and presented as for experimental samples (Canada Safeway, Grade A large, Winnipeg MB)/15g
Milky	Aroma associated with milk	2% milk (Canada Safeway, Lucerne Brand, Winnipeg, MB)/10g
Creamy	Aroma associated with cream	whipping cream (Canada Safeway, Lucerne Brand, Winnipeg, MB)/10g

Buttery	Aroma associated with unsalted butter	unsalted butter (Canada Safeway, Lucerne Brand, Winnipeg, MB)/1g
Salty	Aroma associated with salted butter	salted butter (Canada Safeway, Lucerne Brand, Winnipeg, MB)/1g
Sour	Aroma associated with cultured dairy product	plain low fat yogurt (Canada Safeway, Lucerne Brand, Winnipeg, MB)/10g

Flavour

Egg	Flavour associated with scrambled egg	blended commercial egg cooked and presented as for experimental samples (Canada Safeway, Grade A large, Winnipeg MB)/15g
Milky	Flavour associated with milk	2% milk (Canada Safeway, Lucerne Brand, Winnipeg, MB)/5g
Creamy	Flavour associated with cream	whipping cream (Canada Safeway, Lucerne Brand, Winnipeg, MB)/5g
Buttery	Flavour associated with unsalted butter	unsalted butter (Canada Safeway, Lucerne Brand, Winnipeg, MB)/5g
Salty	Flavour associated with sodium chloride	0.25g coarse salt (Sifto Canada Inc. Mississauga, ON) in 100g filtered water
Sour	Flavour associated with citric acid	0.02g citric acid (Rougier Pharma, Mirabel, QC) in 100g filtered water
Sweet	Flavour associated with sucrose	1g sucrose (Rogers, Lantic Inc., Montreal, PQ) in 100g filtered water

[1]Placed in 60mL plastic portion cup and capped with plastic lid about 1 hour prior to evaluation served at room temperature; except for butter samples at 4°C.

On the days of sensory analysis, samples were prepared as described above (Section 2.6). All sensory evaluations were conducted in individual partitioned work stations at the sensory laboratory at the University of Manitoba. Work stations were equipped with SIMS 2000 (2010) computerized sensory software (Sensory Integrated Management System, Morristown, NJ, U.S.A.). Light from incandescent bulbs directed through red opaque plastic was used in work stations to mask any potential colour differences between samples. Samples were coded with randomly selected 3-digit numbers and order of tasting between and within days was balanced to account for first order and carry-over effects. The aroma and flavour attributes of different samples were scored on unstructured 15 cm line scales from 0 (low) to 15 (high). Each panelist

was provided with filtered, room temperature water and an unsalted cracker to cleanse his or her palate between tastings. Panelists' evaluated aroma attributes first, followed by flavour attributes (See Appendix O). Eggs from each of the six dietary treatments were replicated three times on three separate days within the same week. At each of the three egg tasting sessions, panelists assessed six samples.

Figure 4.2: Picture of work station in sensory laboratory
Photograph by © Erin Goldberg

4.3.9. Colour analysis

Yolk colour was determined for eggs collected from hens at 29 weeks of age (Week 10 of the experiment). Egg whites were separated from yolks and discarded. Yolks (8 per treatment)

were gently mixed in a bowl, ensuring there were no bubbles. Any chalaza remaining in the

mixed yolk were removed with a strainer and discarded. A Hunter Lab colourimeter (MiniScan

EZ) calibrated to the white tile for L*a*b* was used to determine yolk colour. Illuminant D65,

which corresponds to average daylight, was the source of light for this instrument. Yolk (16 ±

0.05g) was weighed into each of 3 Petri dishes (1007 Petri dish, 60 x 15mm). Readings were

taken in triplicate.

Figure 4.3: Picture of Hunter colourimeter
Photograph by © Erin Goldberg

4.3.9.1. Statistical analysis

　　This study was designed as a completely randomized study. Sensory data was analyzed

using three-way analysis of variance (ANOVA) using PASW Statistics 18.0.3. The model

included the following random effects: Dietary Treatments (T), Panelists (P), and Replications (R), and two-way interactions of Panelist by Dietary Treatment, Dietary Treatment by Replication and Panelist by Replication. Nonsignificant interactions were pooled, according to the methods outlined in O'Mahony's Sensory Evaluation of Food (pp. 230-231) and Fisher's least significant difference (LSD) test was used to determine mean treatment differences when significant (P<0.05). Fatty acid and yolk colour data were analyzed using one-way ANOVA following multiple comparisons using Tukey's test. Bi-plots were generated using partial least squares (PLS) (XLSTAT version 2011) using average values for all of the attributes to provide a visual perspective of the correlation between the samples in relation to the corresponding yolk FA and sensory attribute intensities.

4.4. Results and Discussion

4.4.1. Bird health

Data on the performance and egg quality of the hens consuming diets containing either HS or HO has been reported previously (Gakhar, Goldberg, House 2010). In brief, the inclusion of HS or HO did not significantly affect measures of egg production or feed efficiency, and average feed consumption values were between 90-100 g/d.

4.4.2. Fatty acid composition of egg yolks

Yolk fatty acid analysis from all diets is shown in Table 4.3. Dietary treatments had no effect on the content of PALM, PALMO, LA, AA, or total n-6 PUFA in the egg yolk. Stearic acid (SA) slightly increased and OA slightly decreased with increasing dietary HO or HS (P<0.001). Yolk content of ALA and total n-3 PUFA increased with increasing dietary HO,

reaching a peak of 11.6mg ALA/g of yolk, 0.2mg EPA/g of yolk and 15.3mg total n-3 PUFA/g

of yolk in the 12% HO treatment group. The level of yolk GLA, EPA, DPA and DHA were also

increased in HO groups compared to the control but not to the same extent. Yolk content of

ALA and total n-3 PUFA increased with increasing dietary HS, reaching a peak of 6.0mg ALA/g

of yolk and 9.3mg total n-3 PUFA/g of yolk in the 20% HS treatment group. GLA, EPA, DPA

and DHA increased in the 20% HS group compared to the control, but were not significantly

different from the 10% HS group.

Table 4.3: Fatty acid composition of egg yolks from hens consuming hempseed oil or hempseed

Fatty Acid (mg/g yolk)	0% H	4%HO	8%HO	12%HO	10% HS	20% HS	SEM	P-Value
				Treatment				
PALM	48.6	46.7	44.7	43.9	48.0	45.5	0.61	NS
PALMO	1.9	1.9	2.1	2.0	2.2	1.8	0.05	NS
SA	17.8^a	19.1^{ab}	20.3^{bc}	22.2^c	19.8^{ab}	20.0^{abc}	0.28	***
OA	65.2^c	60.3^{bc}	55.2^{ab}	48.0^a	63.2^{bc}	54.2^{ab}	1.19	***
LA	63.2	63.7	60.7	66.1	64.5	67.6	0.78	NS
GLA	0.4^a	0.5^{ab}	0.6^b	0.8^c	0.5^{ab}	0.6^b	0.02	***
AA	4.9	5.0	4.4	4.5	4.7	4.2	0.10	NS
ALA	1.0^a	4.0^b	6.9^c	11.6^d	3.2^b	6.0^c	0.50	***
EPA	0.0^a	0.1^b	0.1^b	0.2^c	0.1^b	0.1^b	0.01	***
DPA	0.1^a	0.4^b	0.4^b	0.5^b	0.3^{ab}	0.3^{ab}	0.03	***
DHA	1.2^a	3.0^{bc}	3.1^c	3.0^c	2.4^b	2.9^{bc}	0.12	***
Total n-6	68.5	69.2	65.7	71.4	69.7	72.4	0.81	NS
Total n-3	2.4^a	7.5^c	10.6^e	15.3^f	6.0^b	9.3^d	0.60	***

Data within a row with different superscripts are significantly different.
(NS P≥0.05; ***P<0.001)
H= Hemp; HO= Hempseed oil; HS= Hempseed; GLA= Gamma-linolenic acid;
AA= Arachidonic acid; ALA= Alpha-linolenic acid; EPA= Eicosapentaenoic acid;
DPA= Docosapentaenoic acid; DHA= Docosahexaenoic acid; Total n-6= Total n-6 fatty acids; Total n-3=
Total n-3 fatty acids.

Although eggs in this study were enriched with higher levels of n-3 PUFA compared to

the control eggs, the degree of enrichment is still not high enough to obtain a nutrient content

claim in Canada, which is at least 300 mg n-3 PUFA per egg (Health Canada 2012b). The

highest level of HO inclusion (12% HO) resulted in eggs with 15.3 mg n-3 PUFA/g of yolk, which is approximately 232 mg of n-3 PUFA per egg. Because such high levels of oil inclusion is likely higher than industry standards due to cost constraints, another feasible option may be to include a lower level of HO or HS in conjunction with other ALA-rich ingredients, such as flax, to increase yolk n-3 PUFA to a level that could obtain status for a nutrient content claim.

Silversides and Le Francois (2005) found that inclusion of 20% HS meal significantly (P<0.05) increased ALA from 0.2% of total FA in the control diet to 1.2%, or by 6 times. In this study, inclusion of 20% HS also increased ALA content in the yolk by 6 times, however, ALA accounted for 3% of total FA. The 8 and 12% HO diets increased ALA content to 3.5 and 5.8% of total FA, respectively. Even the lowest level of HO or HS inclusion in this study resulted in yolks with a greater percentage of ALA (2%) compared to the 20% HS meal diet in the study by Silversides and Le Francois (2005). Therefore, utilizing the seed or oil as compared to the meal alone, as shown in this study, resulted in even greater changes in yolk fatty acid profile.

4.4.3. Sensory analysis

Aroma and flavour definitions and standards used during training are shown in Table 4.2 and the results from sensory analysis in Table 4.4. Results of ANOVA showed for all attributes tested, there was a highly significant difference observed over the range of scores by individual panelists (P<0.01), which is expected because individuals may have their own frame of reference, despite the attempt to decrease this gap during training sessions. Similarly, there were differences seen across testing days for some but not all sensory attributes. For example, salty aroma was highly significantly different (P<0.001) across the 3 days, as were milky and creamy aroma (P<0.05). For flavour attributes, only sweet and sour were significantly different across

treatment days (P<0.05). This suggests that certain attributes were more difficult for panelists to detect in a consistent manner over different sessions. Due to the significant differences seen in panelists and replications, this caused the interaction effects seen between panelist and replication. In all samples, the most intense aroma and flavour attribute was described as 'egg', and the least intense attribute was 'sour'. Also, no significant differences in aroma and flavour attributes between treatments for cooked egg samples were found, with the exception of sweet flavour. Eggs from the 4% HO group were the least sweet, whereas eggs from the 20% HS group were the sweetest and these two groups were significantly different from each other. One possible explanation for this could have been due to the ratio of yolk to white in these samples. Warren, Larick and Ball (1995) tested egg yolk, white, and combinations of the two for sensory attributes. They found that 58:42 ratio samples had a sweeter flavour compared to 10:90 ratio samples (yolk:white, respectively). However, eggs used for cooking in the current study were weighed to ensure eggs of similar weight were used in order to avoid this. Even if there happened to be less yolk in the 4% HO samples, the difference due to this factor is probably negligible as Warren, Larick and Ball (1995) found that a sample would have to contain mostly egg white in order for this to occur.

Table 4.4: Descriptive analysis results from three-way analysis of variance (T=Dietary Treatment (n=6); P=Panelist (n=8); Replication (n=3)) and Fisher's least significant difference test for cooked eggs from hens consuming hempseed oil or hempseed

Attribute	Source of variation (F-value)						Treatment (mean intensity ratings – 0 to 15 cm line scale)					
	T	P	R	P x T	T x R	P x R	0% H	4% HO	8% HO	12% HO	10% HS	20% HS
Aroma												
Egg	1.45 NS	47.90 ***	1.72 NS	1.04 NS	0.89 NS	1.08 NS	8.0	7.3	7.1	7.9	7.9	8.0
Milky	1.09 NS	19.45 ***	4.09 *	0.77 NS	0.77 NS	3.83 ***	3.0	2.5	2.8	3.1	3.2	3.1
Creamy	0.92 NS	13.27 ***	4.61 *	0.92 NS	1.08 NS	1.29 NS	2.5	2.3	1.9	2.5	2.2	2.6
Buttery	0.26 NS	3.21 **	1.13 NS	0.85 NS	0.45 NS	0.82 NS	2.3	2.3	2.1	2.1	2.1	2.2
Salty	0.50 NS	8.86 ***	8.28 ***	0.73 NS	0.83 NS	4.27 ***	2.4	2.4	2.6	2.3	2.2	2.4
Sour	0.87 NS	20.42 ***	2.77 NS	0.92 NS	0.84 NS	2.63 **	1.0	0.7	0.7	0.8	1.0	0.8
Flavour												
Egg	0.99 NS	58.32 ***	1.65 NS	0.94 NS	1.35 NS	1.14 NS	7.1	7.1	7.4	8.0	7.3	7.3
Milky	0.47 NS	7.35 ***	0.07 NS	0.97 NS	1.22 NS	2.56 **	2.8	2.5	2.7	2.8	3.1	2.9
Creamy	0.77 NS	7.35 ***	0.52 NS	1.00 NS	1.37 NS	1.62 NS	2.3	2.5	1.9	2.2	2.6	2.1
Buttery	0.83 NS	15.06 ***	0.38 NS	2.74 ***	0.93 NS	3.08 ***	1.9	2.2	1.7	1.9	2.0	1.9
Salty	1.04 NS	21.19 ***	2.33 NS	0.99 NS	0.92 NS	3.03 ***	1.5	1.2	1.3	1.6	1.5	1.6
Sour	2.10 NS	19.22 ***	4.61 *	0.94 NS	0.83 NS	3.59 ***	0.3	0.5	0.4	0.8	0.4	0.4
Sweet	2.43 *	54.16 ***	5.62 **	0.75 NS	1.38 NS	3.39 ***	2.3[ab]	1.8[a]	1.9[ab]	2.3[ab]	2.3[ab]	2.4[b]

Means across treatments with different superscripts are significantly different from each other. (NS p≥0.05; *P<0.05, **P<0.01, ***P<0.001)

H= Hemp; HO= Hempseed oil; HS= Hempseed

Van Elswyk, Dawson and Sams (1995) found that eggs from hens fed 0.5% menhaden fish oil tasted the least sweet compared to 0%, 1.5%, or 3.0% MO treatments. In fact, sweet taste was one of only five sensory attributes that were significantly influenced by dietary treatment, with three of them being related to 'fishy' notes. Interestingly, in the current study, eggs from the lowest level of HO inclusion tasted the least sweet. It seems that a low level of dietary n-3 PUFA affected the degree of sweetness, or is potentially masked by other flavours, whereas a higher level or none at all results in no difference.

An important aspect of this research was the effect feeding hemp to hens had on the sensory quality of eggs. Previous research has demonstrated that when dietary levels of flaxseed in the hen diet exceed 10%, there is possibility for overall acceptability of the eggs to decrease compared to non-enriched eggs (Leeson, Caston, MacLaurin 1998). Since PUFA in particular are more susceptible to oxidation, it is important that an adequate level of antioxidants be incorporated into diets to prevent lipid oxidation from occurring. Vitamin E is a commonly used antioxidant in animal diets. However, additional vitamin E beyond what is normally used in poultry feed was incorporated in the current study's diets to account for the high level of total lipid, in particular n-3 PUFA, rather than to ameliorate sensory changes in the resulting eggs. In fact, in a study conducted by Leeson, Caston and MacLaurin (1998), it was found that untrained panelists did not perceive an off-flavour (P>0.05) in eggs from hens fed 20% flaxseed and 10 IU/kg vitamin E compared to eggs from hens fed the same amount of flaxseed, but with ten times the amount of vitamin E in the diet. However, the flaxseed fed to the hens in this study were whole, and not ground, therefore chances of lipid oxidation occurring to an extent to alter sensory profiles would be minimized. Despite these findings from their first experiment, their subsequent findings suggested such high levels of vitamin E (100 IU/kg) did in fact result in eggs

with lower acceptability. Although the HO and HS used in the current diets may be more vulnerable to oxidation compared to the diets in Lesson and others (1998), it is reasonable to suggest that the lipids in the feed were sufficiently preserved due to the incorporation of additional vitamin E (150 IU/kg) in the hemp treatment diets, beyond that which was used in the former study. In addition, because panelists did not perceive any other sensory attributes in the eggs other than the attributes given in Table 4.2, it is unlikely the level of dietary vitamin E used in the current study's diets had a significant sensory impact. A consumer panel may be necessary in order to fully elucidate this conclusion by determining overall acceptability. Although the level of total lipid in this study's diets was too high to be realistically used in industry, that being approximately 13%, this study suggests that extremely high levels of HS and HO could be used safely in hen feed resulting in no negative sensory impact. Because HS and HO are not high enough in n-3 PUFA to result in deleterious sensory changes in eggs, egg producers need not be concerned with using hemp products as feed ingredients.

The cooking method used in this study has not been utilized in past sensory experiments. Usually eggs are presented to panelists in a scrambled or hard-boiled form, as consumers most often use these preparation methods in cookery. However, this new cooking method offers a number of advantages over others. The first advantage is that samples are more consistently cooked and temperature held across treatments. The temperature of the sample can be determined easily using a digital thermometer; thereby ensuring samples are cooked properly. Lastly, the use of the aluminum lid on top of the sample enable volatile compounds produced during the cooking process to be retained in the sample; therefore panelists may be able to detect sensory changes more easily. Therefore, it is highly unlikely that there are any sensory changes

in eggs from hens fed HO or HS if eggs are cooked using a conventional method, where volatiles escape more easily.

Descriptive analysis tests using the same product cooked using two different methods have revealed some sensory differences. For example, in eggs from hens fed MFO, there were no significant sensory differences between treatments for scrambled egg samples, but boiled egg samples from the 6.0% MFO diet treatment had significantly higher "off-flavour" and "sulphur-flavour" (Lawlor and others 2010). One explanation for this could be explained by differences in lipid oxidation, where Cortinas and others (2003) found oxidation levels of hard-boiled eggs to be 30.4% higher compared to scrambled eggs. However, they also reported that addition of 100mg/kg α-tocopheryl acetate in the hen diet reduced lipid oxidation associated with cooking eggs enriched in n-3 LCPUFA. Contrary to findings by Lawlor and others (2010), Van Elswyk and others (1992) reported that panelists differentiated n-3 enriched eggs from controls (P<0.01) when scrambled but not when hard cooked. However, this study utilized a consumer panel using a duo-trio test, compared to a trained panel using a descriptive test. Therefore, making comparisons between these two methods is not appropriate. The author proposed that increased 'fishy' notes in the scrambled eggs could have been the result of increased availability of volatile flavour compounds due to serving the samples heated. Despite conflicting results, differences in ingredients, feed antioxidant levels and method of incorporation as well as cooking method and presentation method to panelists could have all influenced the sensory outcomes.

The graphical presentation of the correlation loadings (Figure 4.4) shows overall possible relations between specific yolk FA and aroma and flavour attributes of cooked eggs. There are a few important observations to note about this visual depiction of the data correlation. The first, and the most notable, is the presence of OA in relation to the n-3 PUFA, GLA and SA. Also,

falling into the latter cluster of FA was the 12% HO group, which happens to be the group containing the highest yolk concentration of all of these noted FA. Based on the degree of separation between OA and egg flavour, it is possible that yolk OA may be associated with decreased egg flavour intensity. Moreover, greater n-3 PUFA, GLA and SA yolk concentration may be related to increased egg flavour intensity. Oleic acid is potentially more related to creamy and buttery flavour. Creamy and buttery aroma and flavour appear to be closely related, along with the 10% HS group. The 8% HO is closely related to salty aroma. Finally, corresponding with the results from sensory analysis, the only attribute that was significantly different was sweet flavour, with the 4% HO group bearing the least sweet eggs. When analyzing the bi-plot, it can be seen that sweet flavour and the 4% HO group are on opposite ends of the axis, inferring a distinct difference.

Correlation Loadings (X and Y)
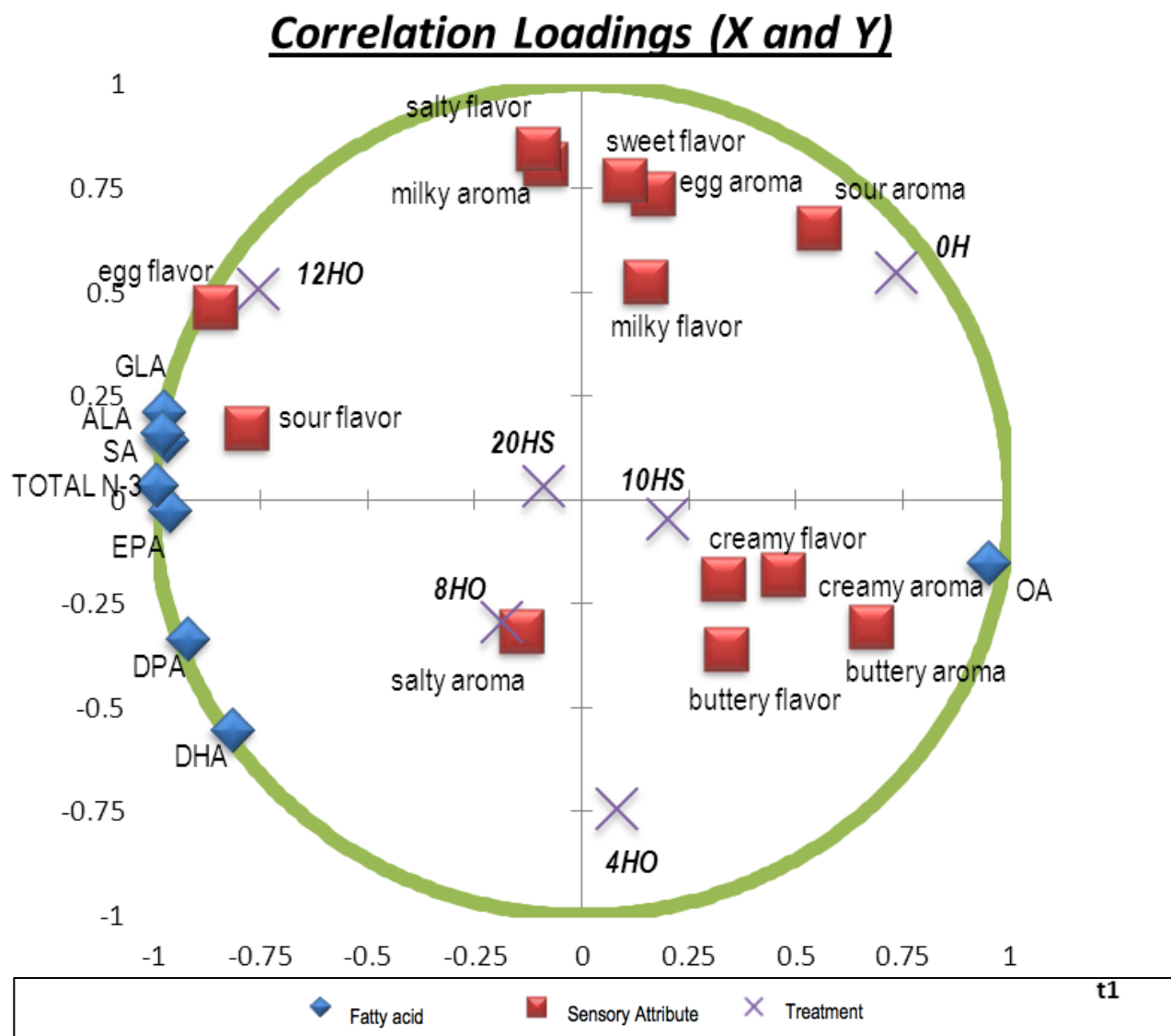
Figure 4.4: An overview of the correlation loadings from partial least squares analyses with yolk fatty acids as X-variables and sensory attributes of cooked egg samples as Y-variables from hens consuming hempseed oil or hempseed

H= Hemp; HO= Hempseed oil; HS= Hempseed; SA= Stearic acid; OA= Oleic acid; GLA= Gamma-linolenic acid; ALA= Alpha-linolenic acid; EPA= Eicosapentaenoic acid; DPA= Docosapentaenoic acid; DHA= Docosahexaenoic acid; Total n-3= Total n-3 fatty acids.

4.4.4. Colour analysis

Results of yolk colour analysis are shown in Table 4.5. L*, a* and b* values (Mean

±SEM) for control eggs were 61.2±0.10, 1.1±0.05, and 43.0±0.22, respectively. Addition of

either HS or HO led to significant (P<0.05) reductions in L*, and significant (P<0.05) increases

in a* and b*, with the largest changes observed in the 20% HS treatment (L*=58.7; a*=5.8;

b*=60.5). This means that as the concentration of HO or HS in the diet increased, the colour of

the corresponding yolk increased in darkness, yellowness and redness. The degree of yellowness

and redness were more influenced by the changes in diet compared to the degree of lightness.

Table 4.5: Colour analysis of egg yolks from hens consuming hempseed oil or hempseed

Treatment	L*	a*	b*
0% H	61.2^e	1.1^a	43.0^a
4% HO	60.8^{de}	1.9^b	46.5^b
8% HO	60.4^{cd}	2.4^c	47.7^c
12% HO	59.6^b	3.4^d	51.9^d
10% HS	60.3^c	3.4^d	52.3^d
20% HS	58.7^a	5.8^e	60.5^e
F-Ratio	74.05	1169.96	748.85
SEM	0.10	0.05	0.22
P-Value	***	***	***

Data within a column with different superscripts are significantly different.
(NS P≥0.05; ***P<0.001)
H= Hemp; HO= Hempseed oil; HS= Hempseed

The intensity of yolk colour is mainly due to the presence of yellow and red

oxycarotenoids, or xanthophyll pigments, in the hen diet (Karunajeewa and others 1984).

Depending on the level of these compounds in the diet, yolk colour can range from almost no

colour to a deep orange. Yolk colour intensity has been shown to influence the perception of

quality, which may lead to preferences for certain yolk colour. For instance, panelists scored

ordinary eggs with a pale yolk low for acceptability compared to more pigmented enriched eggs

based on appearance (Farrell 1998). Conversely, Ofosu and others (2010) found that panelists

had a higher preference for egg yolks with lighter colour compared to a more intense colouring. It is important to note that although the colour changes in the yolk were statistically significant ($P<0.001$), the perception to the human eye may be relatively insignificant, and these results cannot infer what consumer preferences may be. In fact, sensory panelists could not differ between treatments based on colour during training sessions where red lighting was not utilized.

Additionally, not only has yolk colour been shown to influence the degree of consumer acceptability, it may also be indicative of minor changes in yolk micronutrient content. More desirable carotenoids include β-carotene, which has nutritional value as a precursor to vitamin A, and lutein and zeaxanthin, which exhibit antioxidant function and contribute to eye health (Carpentier, Knaus, Suh 2009). Hempseed receives its characteristic green colour from a mixture of carotenoids, chlorophylls, and xanthophylls (Latif and Anwar 2009); therefore HO and HS may be considered useful in providing natural pigments to hen feed in order to enhance yolk colour, as well as the nutritional composition of eggs. Because carotenoid absorption is increased in the human intestine when consumed with lipids, eggs may act as an effective delivery system for some carotenoids (Olson, Ward, Koutsos 2008). The concentration and profile of carotenoids in the yolks should be determined in order to confirm that desirable carotenoids are significantly increased. Additionally, further analyses using other strains of laying hen is necessary in order to conclude the effect of hemp ingredients on yolk colour, as hen genetics may also affect yolk colouration.

4.5. Conclusions

The present study's objectives were to test the efficacy of utilizing HS and HO in hen diets to enrich eggs in n-3 PUFA, and to determine the sensory changes in these eggs. This was

addressed by testing egg yolks for fatty acid content and colour, and by utilizing a sensory panel to determine the aroma and flavour of cooked eggs.

The use of HS at levels up to 20% and HO at levels up to 12% in the laying hen diet lead to significant increases in the ALA and total n-3 PUFA content of eggs. Despite the increased level of n-3 PUFA in the eggs, there were no sensory differences in cooked eggs across all dietary treatments, with the exception of sweet flavour, where minor differences were observed. Egg flavour appeared to be negatively correlated with OA, and positively correlated with n-3 PUFA, GLA and SA. This suggests that hemp incorporation into hen feed may increase the egg flavour intensity in eggs procured from these hens due to increases in n-3 PUFA, GLA and SA. It is important to note that the n-3 PUFA levels found in eggs from any of the treatments may not be high enough to be labeled as 'n-3' in certain marketplaces, such as in Canada where n-3 eggs must contain at least 300mg n-3 PUFA per 50 g. egg. However, HS and HO could be used in combination with other high ALA ingredients such as flaxseed in order to obtain such levels. Further research into diet mixtures of hemp and flax to produce commercially labeled 'n-3' eggs is needed, including sensory evaluation and carotenoid determination of yolks. In addition, sensory analysis of stored eggs from hens fed hemp products is necessary to determine if storage has a significant impact on sensory attributes. In summary, this study has shown that it is possible to incorporate very high levels of HO or HS in hen diets to increase n-3 PUFA and enhance yolk colour all without negatively affecting the sensory quality in fresh table eggs.

4.6. Acknowledgment

CHAPTER 5: FATTY ACID PROFILE AND SENSORY CHARACTERISTICS OF TABLE EGGS FROM LAYING HENS FED DESIGNER DIETS

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5.1. Abstract

The fatty acid composition of eggs is highly reflective of the diet of the laying hen. Therefore, nutritionally important FA can be increased in eggs in order to benefit human health. To explore the factors affecting the hens metabolism and deposition of FA of interest, the current research was divided into 2 studies. In Study 1, the fatty acid profile of eggs from Bovan White hens fed either 8%, 14%, 20% or 28% of the n-6 FA, LA (expressed as a percentage of total FA), and an additional treatment of 14% LA containing double the amount of SFA was determined. Omega-6 PUFA and DPA in the yolk were significantly ($P<0.05$) increased, and oleic acid and EPA were significantly decreased with an increasing dietary LA content. In Study 2, the FA and sensory profiles were determined in eggs from Shaver White hens fed either 1) 15% or 30% of ALA (of total FA), and 2) low (0.5), medium (1) or high (2) ratios of SFA: LA + OA. Increasing this ratio resulted in marked increases in lauric acid (LAUR), ALA, EPA, DPA, DHA with decreases in LA and AA. Increasing the dietary ALA content from 15% to 30% (of total FA) did not overcome the DHA plateau observed in the yolk. No significant differences ($P\geq0.05$) in aroma or flavour between cooked eggs from the different dietary treatments were observed among trained panelists (n=8). The results showed that increasing the ratio of SFA:LA+OA in layer diets has a more favorable effect on the yolk FA profile compared to altering the LA content at the expense of OA, all while maintaining sensory quality.

5.2. Introduction

Linoleic acid (LA) and ALA are essential FA found abundantly in plant sources that must be obtained in the human diet. LA and ALA are the parent compounds of the n-6 and n-3 PUFA series, respectively. The health benefits of consuming n-3 PUFA are well established (Yashodhara and others 2009). In particular, consumption of n-3 LCPUFA like EPA, DPA and DHA, typically found in fish and algae, offer cardio-metabolic, immunologic and neurologic protection, as reviewed by Innis, Novak and Keller (2012). The current recommendation to prevent deficiency symptoms is between 0.6-1.2% n-3 PUFA of total energy, with up to 10% of this range composed of EPA and DHA (Health Canada 2012c). Humans have the ability to convert ALA into n-3 LCPUFA through a series of desaturation and elongation enzymatic reactions. Specifically, D6DE and D5DE enzymes (EC 1.14.19.3) are necessary to complete the process. However, conversion efficiency of ALA decreases down the elongation-desaturase cascade; therefore DHA synthesis from ALA (~0.5%) is even more restricted than that of EPA (~5%) (Goyens and others 2006; Plourde and Cunning 2007; Brenna and others 2009). Furthermore, higher conversion rates in women have been reported, possibly due to the activation of the peroxisomal pathway by estrogens (Giltay and others 2004; Burdge 2004).

The n-6:n-3 PUFA ratio in the diet plays an important role in health and disease (Myers and Allen 2012). The mechanism behind this can be attributed to the pro-thrombotic, pro-aggregatory, and pro-inflammatory properties of the eicosanoid metabolic products of n-6 PUFA as compared to the opposing physiological functions of n-3 PUFA, therefore reducing this ratio in the diet is favorable (de Lorgeril and Salen 2012; Simopoulos 2002, 2008). Additionally, it has been found that there are several dietary factors which affect the conversion of ALA to LCPUFA, including the LA:ALA ratio. It is suggested that because LA and ALA compete for

the same rate-limiting enzymes, D5DE and D6DE, decreasing the LA:ALA ratio is important to encourage greater EPA and DHA synthesis (Simopoulos 2010). However, others have suggested that the absolute amount of dietary ALA is more indicative of the degree of ALA conversion rather than the ratio (Goyens and others 2006).

As consumption of n-3 PUFA in the Western diet is limited, particularly LCPUFA, developing alternative ways to increase consumption is critical. The use of hen eggs to serve this function is attractive for several reasons. Most importantly, the hen liver is able to convert a substantial proportion of dietary ALA into LCPUFA that can be deposited in the egg yolk. Because of this, vegetarian ingredients can be used in the feed rather than unsustainable marine products which may also be contaminated with mercury (Health Canada 2012a). In harnessing the hen's natural ability to convert ALA to LCPUFA, this functional food could serve a critical role in the health of individuals who do not consume seafood for various reasons. Additionally, it is well-known that the incorporation of n-3 PUFA in eggs may result in the development of undesirable aroma and flavour attributes. Therefore, sensory evaluation is an essential component in determining any potential differences in these eggs.

The majority of studies in this area have focused on the resulting egg fatty acid composition through the inclusion of particular ingredients, such as fish, algae or oilseeds, rather than controlling for dietary effects by manipulating the dietary fatty acid composition. Our previous work has demonstrated that a decreased LA:ALA ratio in the layer diet results in increased yolk LCPUFA, eventually reaching a DHA plateau (Goldberg and others 2012). Therefore, the objectives of this research were to incorporate oils and fats in varying amounts in order to maximize ALA conversion and deposition of FA of interest into resultant eggs while assessing the potential for any sensory changes that may occur.

5.3. Materials and Methods

This research was divided into two Studies. The objectives of each study are listed below:

1) Study 1 – To determine the impact of LA in the layer diet on yolk fatty acids.

2) Study 2 – To determine the impact of SFA in the layer diet on yolk fatty acids.

The main purpose of both studies was to determine the extent to which these dietary fats influence the conversion of ALA to DHA into egg yolk if competition between n-3 and n-6 PUFA for access to D5DE and D6DE is removed. By maintaining a constant ALA level in our diets in both Studies 1 and 2, we were able to assess this more effectively. Additionally, it is important that sensory quality be maintained in order to remain palatable to consumers; therefore, it would be a desired outcome if the aroma and flavour of these eggs remain unaffected despite the expected fatty acid alteration.

5.3.1. Bird housing and environment

Forty Bovan White hens at 49 weeks of age and forty-eight Shaver White laying hens at 37 weeks of age (Steinbach Hatchery & Feed Ltd., Steinbach, MB, Canada) were used in Studies 1 and 2, respectively. Hens from both studies were housed individually according to the methods described in our previous work (Goldberg and others 2012), with approval from The University of Manitoba's Animal Care Protocol Review Committee, in accordance with recommendations established by the Canadian Council on Animal Care (Canadian Council on Animal Care 1993).

5.3.2. Diets

The isoenergetic and isonitrogenous diets used in Studies 1 and 2 are shown in Tables 5.1 and 5.2, respectively.

Table 5.1: Ingredient composition of layer diets used to determine the impact of altering the dietary linoleic acid content on egg yolk lipids

Ingredients (%)	8	14	20	28	14S
Ground wheat	31.82	31.82	31.82	31.82	31.82
Ground soybean meal	23.57	23.57	23.57	23.57	23.57
Ground barley	22.15	22.15	22.15	22.15	22.15
Limestone	10.46	10.46	10.46	10.46	10.46
Canola oil	0.01	3.06	3.14	0.97	1.53
Corn oil	0.00	0.00	0.88	2.73	0.19
Flaxseed oil	4.00	3.51	3.48	3.80	3.72
High oleic sunflower oil	3.49	0.94	0.00	0.00	0.00
Lard	0.00	0.00	0.00	0.00	2.06
VM Premix[1]	2.50	2.50	2.50	2.50	2.50
Dicalcium phosphate	1.59	1.59	1.59	1.59	1.59
Salt	0.32	0.32	0.32	0.32	0.32
Lysine	0.06	0.06	0.06	0.06	0.06
DL-Methionine	0.04	0.04	0.04	0.04	0.04
Calculated Composition					
AMEn (Poultry; Kcal/kg)	2866	2865	2874	2895	2846
Crude Protein (%)	18.50	18.50	18.50	18.50	18.50
Crude Fat (%)	8.88	8.88	8.88	8.88	8.88
Calcium (%)	4.20	4.20	4.20	4.20	4.20
Total Lysine (%)	0.90	0.90	0.90	0.90	0.90
Total Phosphorus (%)	0.67	0.67	0.67	0.67	0.67
Available Phosphorus (%)	0.45	0.45	0.45	0.45	0.45
Sodium (%)	0.15	0.15	0.15	0.15	0.15
Chloride (%)	0.25	0.25	0.25	0.25	0.25
Methionine (%)	0.30	0.30	0.30	0.30	0.30
Threonine (%)	0.65	0.65	0.65	0.65	0.65

8= 8% Linoleic acid; 14= 14% Linoleic acid; 20= 20% Linoleic acid; 28= 28% Linoleic acid; 14S= 14% Linoleic acid + double saturated fat

[1]Provided per kg of diet:11,000 IU vitamin A, 3000 IU vitamin D3, 150 IU vitamin E, 3 mg of vitamin K(as menadione), 0.02 mg cyanocobalamin, 6.5 mg riboflavin, 4 mg folic acid, 10 mg of calcium pantothenate, 40.1 mg niacin, 0.2 mg biotin, 2.2 mg thiamine, 4.5 mg pyridoxine, 1000 mg choline, 125 mg of ethoxyquin (antioxidant), 66 mg Mn (as manganese dioxide), 70 mg Zn (as zinc oxide), 80 mg Fe (ferrous sulfate), 10 mg Cu (as copper sulfate), 0.3 mg Se (as sodium selenite), 0.4 mg I (as calcium iodate), and 0.67 mg iodized salt.

VM= Vitamin-Mineral; AMEn= Nitrogen corrected apparent metabolisable energy

Table 5.2: Ingredient composition of layer diets used to determine the impact of altering the ratio of saturated fat: linoleic and oleic acids and the content of alpha-linolenic acid on egg yolk lipids and sensory quality

	15% ALA[a]			30% ALA[a]		
Ingredients (%)	**L[a]**	**M[b]**	**H[c]**	**L[a]**	**M[b]**	**H[c]**
Ground wheat	31.82	31.82	31.82	31.82	31.82	31.82
Ground soybean meal	23.57	23.57	23.57	23.57	23.57	23.57
Ground barley	22.15	22.15	22.15	22.15	22.15	22.15
Limestone	10.46	10.46	10.46	10.46	10.46	10.46
Corn oil	3.97	2.61	1.25	2.24	1.11	0.00
Flax oil	2.02	2.05	2.08	4.17	4.20	4.22
Coconut oil	1.51	2.84	4.16	1.08	2.20	3.28
VM Premix[1]	2.50	2.50	2.50	2.50	2.50	2.50
Dicalcium phosphate	1.59	1.59	1.59	1.59	1.59	1.59
Salt	0.32	0.32	0.32	0.32	0.32	0.32
Lysine	0.06	0.06	0.06	0.06	0.06	0.06
DL-Methionine	0.04	0.04	0.04	0.04	0.04	0.04
Calculated Composition						
AMEn (Poultry; Kcal/kg)	2886	2871	2857	2852	2840	2828
Crude Protein (%)	18.50	18.50	18.50	18.50	18.50	18.50
Crude Fat (%)	8.88	8.88	8.88	8.88	8.88	8.88
Calcium (%)	4.20	4.20	4.20	4.20	4.20	4.20
Total Lysine (%)	0.90	0.90	0.90	0.90	0.90	0.90
Total Phosphorus (%)	0.67	0.67	0.67	0.67	0.67	0.67
Available Phosphorus (%)	0.45	0.45	0.45	0.45	0.45	0.45
Sodium (%)	0.15	0.15	0.15	0.15	0.15	0.15
Chloride (%)	0.25	0.25	0.25	0.25	0.25	0.25
Methionine (%)	0.30	0.30	0.30	0.30	0.30	0.30
Threonine (%)	0.65	0.65	0.65	0.65	0.65	0.65

L[a], low ratio of saturated fatty acids: linoleic and oleic acids; M[b], medium ratio of saturated fatty acids: linoleic and oleic acids; H[c], high ratio of saturated fatty acids: linoleic and oleic acids.
[a]Expressed as a percentage of total fatty acids.
[1]Provided per kg of diet:11,000 IU vitamin A, 3000 IU vitamin D3, 150 IU vitamin E, 3 mg of vitamin K(as menadione), 0.02 mg cyanocobalamin, 6.5 mg riboflavin, 4 mg folic acid, 10 mg of calcium pantothenate, 40.1 mg niacin, 0.2 mg biotin, 2.2 mg thiamine, 4.5 mg pyridoxine, 1000 mg choline, 125 mg of ethoxyquin (antioxidant), 66 mg Mn (as manganese dioxide), 70 mg Zn (as zinc oxide), 80 mg Fe (ferrous sulfate), 10 mg Cu (as copper sulfate), 0.3 mg Se (as sodium selenite), 0.4 mg I (as calcium iodate), and 0.67 mg iodized salt.
VM= Vitamin-Mineral; AMEn= Nitrogen corrected apparent metabolisable energy.

Diets were formulated to meet the requirements of laying hens consuming 100 g of feed per day (National Research Council 1994). Diets were predominantly a wheat-soybean meal and barley basal mix, using limestone as the calcium source. Diets from Study 1 were prepared by including 8%, 14%, 20%, and 28% LA (expressed as a percentage of total FA). The last treatment group contained 14% LA with double the amount of SFA. All diets were designed to contain 30% ALA (expressed as a percentage of total FA) in order to produce eggs with an n-3 nutrient content claim in Canada, which is at least 300 mg n-3 PUFA per egg (Health Canada 2012b). The changing LA levels in the diet were achieved by varying the designer oil blends in each diet, which consisted of corn, canola, flaxseed and high oleic sunflower oils and the SFA in the last treatment was increased by using lard. Diets from Study 2 were prepared by including 2 levels of ALA: 15 or 30% (expressed as a percentage of total FA), and 3 levels of SFA:LA+OA ratio: low (0.5), medium (1.0) or high (2.0). This was achieved by varying the designer oil blends in each diet, which consisted of corn, flaxseed and nonhydrogenated coconut oil. Due to the high fat content of the diets, an additional 150 international units (IU) alpha-tocopherol (vitamin E) per kg feed was incorporated into all diets as part of the 2.5% vitamin-mineral premix.

5.3.3. Dietary analysis

Feed samples were analyzed for dry matter, crude protein and crude fat according to established procedures (AOAC International 1995). The fatty acid composition of the test diets were determined using standard gas chromatographic techniques of the fatty acid methyl esters (AOAC International 1990), using C17:1 methyl ester as an internal standard.

5.3.4. Experimental protocol

Hens were weighed and caged individually in roll-out style cages, and allowed an

adaptation period of 7 days before being randomly assigned to receive 1 of 5 or 6 dietary

treatments (n=8 per treatment) for Studies 1 and 2, respectively. Both studies were 6 weeks in

duration. Body weights of hens were recorded at the start of the trial, and at weekly intervals.

Feed consumption was determined for the entire week and average daily feed intake and feed

efficiency were calculated. Egg weights were recorded daily and an average egg production rate

was calculated. Prior to sensory analysis, eggs were stored in trays in the dark at 4ºC overnight.

5.3.5. Fatty acid analysis

One egg from each hen at 53 weeks of age and 42 weeks of age was chosen for fatty acid

analysis from Studies 1 and 2, respectively (Week 5 for both Studies).   Egg yolks were

separated from the albumen and stored in plastic bags at -80°C until analysis. Fatty acids were

extracted from the egg yolk according to the methods of Folch, Lees and Stanley (1957). The

fatty acid composition of the yolks was determined in the same manner as the feed.

5.3.6. Sample preparation

Sensory analysis was conducted on eggs from Study 2 only. The cooking method from

Goldberg and others (2012) was used. In brief, four randomly chosen eggs from hens at 43

weeks of age (Week 6 of the experiment) of similar weight (56.0±2.0g) from each treatment

were pooled, mixed, and poured into glass jars (Tradition Inc., Montreal, QC, Canada) and

covered with an aluminum weighing dish (Fisherbrand Cat No. 08-732). Jars were placed on a

metal rack in a stainless steel cooking pot (10 L.) filled with 2L warm water and cooked for

~15±1 min. No cooking oil or salt was used. All prepared samples were analyzed within 10 min

after preparation was complete. Jars were kept at a constant temperature of 55°C in a heated

water bath until panelists evaluated the samples. Advantages of using this cooking method include stable temperature control and retention of volatile compounds produced during the cooking process. Therefore, samples are consistently cooked across treatments and panelists may be able to detect sensory changes more easily.

5.3.7. Recruitment

Eight panelists (5 females, 3 males, aged between 18 and 45) who were students and staff of The University of Manitoba were recruited (See Appendices G-I) to participate in the sensory evaluation. The sensory analysis component of the research received ethical approval from The University of Manitoba's research ethics board (See Appendices C-F). The criteria for participation included open availability, an interest in the panel, and no aversion or allergies to eggs and any ingredients present in any other products to be used for training purposes. Panelist screening for the criteria listed above was conducted through completion of a questionnaire (See Appendix K), where written consent (See Appendix J) was obtained.

5.3.8. Descriptive analysis

The protocol followed for descriptive analysis, which is a modification of the method by Stone and Sidel (2004) can be found in our previous publication (Goldberg and others 2012).   In short, panelists participated in a total of 6 training sessions in order to sufficiently decrease panelist variability (data not shown) before commencing the test sessions. Egg samples were prepared as described above, and presented coded with a randomly selected 3-digit number. During each training session, panelists evaluated the aroma and flavour (See Appendices L-N) of each of the randomized samples and developed an agreed vocabulary of attributes, consisting of

four aroma and four flavour attributes (Table 5.3). Cooked commercial eggs and dairy products

were also evaluated and used as reference points for each attribute.

Table 5.3: Aroma and flavour definitions and standard products used in sensory training sessions for cooked eggs procured from hens consuming different ratios of saturated fat: linoleic and oleic acids and content of alpha-linolenic acid

Attribute		Standard Amount[1]/Amount
Aroma		
Egg	Aroma associated with whole egg	blended commercial egg cooked and presented as for experimental samples (Canada Safeway, Grade A large, Winnipeg MB)/15g
Creamy	Aroma associated with whipping cream	whipping cream (Canada Safeway, Lucerne Brand, Winnipeg, MB)/10g
Buttery	Aroma associated with unsalted butter	unsalted butter (Canada Safeway, Lucerne Brand, Winnipeg, MB)/5g
Sweet	Aroma associated with 2% milk	2% milk (Canada Safeway, Lucerne Brand, Winnipeg, MB)/5g
Flavour		
Egg	Flavour associated with whole egg	blended commercial egg cooked and presented as for experimental samples (Canada Safeway, Grade A large, Winnipeg MB)/15g
Creamy	Flavour associated with whipping cream	whipping cream (Canada Safeway, Lucerne Brand, Winnipeg, MB)/5g
Buttery	Flavour associated with unsalted butter	unsalted butter (Canada Safeway, Lucerne Brand, Winnipeg, MB)/5g
Sweet	Flavour associated with 2% milk	2% milk (Canada Safeway, Lucerne Brand, Winnipeg, MB)/5g

[1]Placed in 60mL plastic portion cup and capped with plastic lid about 1 hour prior to evaluation served at room temperature; except for butter samples at 4°C.

On the days of test panels, evaluations were conducted individual partitioned work

stations equipped with SIMS 2000 (2010) computerized sensory software (Sensory Integrated

Management System, Morristown, NJ, U.S.A.) at the sensory laboratory at The University of

Manitoba. Additionally, red lighting was used in work stations to mask any potential colour

differences between samples. The order of tasting between and within days was balanced to

account for first order and carry-over effects. The sensory attributes of different samples were scored on unstructured 15cm line scales from 0 (low) to 15 (high). Each panelist was provided with filtered, room temperature water to cleanse his or her palate between tastings. Panelists' were directed to evaluate aroma attributes first, followed by flavour attributes (See Appendix O). Egg evaluations were replicated three times on three separate days within the same week, and six samples were assessed at each of the tasting sessions.

5.3.9. Statistical analysis

Both studies were designed to be completely randomized, and all analyses were conducted using SPSS Statistics version 20.0 (SPSS Inc., Chicago, IL). Fatty acids were analyzed using one-way and two-way ANOVA for Studies 1 and 2, respectively. Sensory data were analyzed using three-way ANOVA. The model included Dietary Treatments (T) and Panelists (P) as fixed effects and Replications (R) as a random effect, and two-way interactions of Panelist by Dietary Treatment, Dietary Treatment by Replication and Panelist by Replication. When interactions were not significant they were pooled with the error (O'Mahony 1986). F values were recalculated with the additional sums of squares for error and the corresponding degrees of freedom. If a significant panelist-by-treatment interaction was observed, the main effects were tested by the interaction effect (See Appendix P) (Stone, Bleibaum, Thomas 2012). Fisher's LSD test was used to determine mean treatment differences when significant ($P<0.05$). PLS (XLSTAT version 2012 Addinsoft, Paris, France) analysis was used to generate a biplot by using average values for all attributes of interest to provide a visual perspective of the correlation between the samples in relation to the corresponding yolk FA and sensory attribute intensities.

5.4. Results and Discussion

5.4.1. Bird health

Mean values across treatments including the control from each of Studies 1 and 2 were not significantly different from one another. The following data are expressed as mean values ± the standard error of the mean (SEM) for Study 1 and 2, respectively. Egg production per week was 6.2 ± 0.10 and 6.6 ± 0.09, egg weight was 62.6 ± 0.61 g and 58.4 ± 0.45 g, average feed consumption was 90.8 ± 1.58 g/day and 97.0 ± 1.56 g/day, feed conversion efficiency was 1.45 ± 0.02 g of feed/g of egg and 1.67 ±0.03 g of feed/g of egg. Birds from Study 1 initially weighed 1.8 ± 0.01 kg, and by the end of the trial they weighed 1.7 ± 0.04 kg. Birds from Study 2 initially weighed 1.7 ± 0.01 kg, and by the end of the trial they weighed 1.6 ± 0.02 kg.

5.4.2. Fatty acid composition of egg yolks

Yolk fatty acid analysis from Studies 1 and 2 are shown in Tables 5.4 and 5.5, respectively. In Study 1, n-6 PUFA, OA, EPA and DPA were affected by dietary treatment. An increasing level of dietary LA led to a decrease in yolk OA while LA, GLA, AA and total n-6 PUFA were increased. This observation was expected because LA in the diet is increased at the expense of OA. Increasing LA in the diet also led to a decrease in EPA with an increase in DPA. Doubling the SFA content of the 14% LA treatment did not affect the yolk fatty acid content. The increase in DPA was rather unexpected as our hypothesis was that suppression of dietary LA would lead to a greater amount of ALA conversion due to preferential desaturation of ALA. Furthermore, we also expected to see a decrease in yolk DHA as LA increased; however, the differences between treatments in dietary LA were not large enough to change DHA levels. Altering the ratio of n-6:n-3 PUFA or the absolute amount of ALA might have a more profound

effect, as we have found in a previous work (Goldberg and others 2012), mainly due to the larger differences in ALA content. Study 2 attempted to answer this question while proposing an alternative method to alter the dietary fatty acid composition.

Table 5.4: Fatty acid composition of egg yolks from hens consuming diets with different linoleic acid contents

Fatty Acid (mg/g yolk)	Treatment					F-Ratio	*P*-Value
	8	14	20	28	14S		
PALM	45.1	44.2	44.7	44.9	43.9	0.06	NS
PALMO	6.4	6.0	5.7	5.4	5.5	1.64	NS
SA	15.3	17.0	16.4	18.7	16.8	1.50	NS
OA	99.0^b	93.2^{ab}	85.3^{ab}	83.2^{ab}	79.6^a	3.60	*
LA	24.4^a	28.8^{ab}	35.1^{bc}	36.3^c	29.8^{abc}	8.28	***
GLA	0.2^a	0.3^{ab}	0.3^{ab}	0.4^b	0.3^{ab}	3.76	**
AA	2.3^a	2.5^{ab}	2.8^{ab}	3.0^b	2.5^{ab}	3.07	*
ALA	9.5	9.9	10.2	9.2	10.0	0.44	NS
EPA	0.3^b	0.3^b	0.3^b	$0.2a$	0.3^b	8.53	***
DPA	0.4^a	0.4^a	0.5^{ab}	0.6^b	0.5^{ab}	3.12	*
DHA	4.2	3.7	3.9	4.4	3.9	1.77	NS
TOTAL n-6	27.0^a	31.5^{ab}	38.3^{bc}	39.6^c	32.5^{abc}	7.94	***
TOTAL n-3	14.4	14.4	14.8	14.4	14.7	0.07	NS

8= 8% Linoleic acid; 14= 14% Linoleic acid; 20= 20% Linoleic acid; 28= 28% Linoleic acid; 14S= 14% Linoleic acid + double saturated fat

Data within a row with different superscripts are significantly different.
(NS $p \geq 0.05$; *$P<0.05$, **$P<0.01$, ***$P<0.001$)

PALM= C16:0; PALMO= C16:1; SA= C18:0; OA= C18:1n9; LA= C18:2n6; GLA= C18:3n6; AA= C20:4n6; ALA= C18:3n3; EPA= C20:5n3; DPA= C22:5n3; DHA= C22:6n3; TOTAL n-6= C18:2n6 + C18:3n6 + C20:4n6; TOTAL n-3= C18:3n3 + C20:5n3 + C22:5n3 + C22:6n3.

Table 5.5: Fatty acid composition of egg yolks from hens consuming diets with different ratios of saturated fat: linoleic and oleic acids and content of alpha-linolenic acid

| Fatty Acid (mg/g yolk) | Treatment | | | | | | SEM | P-Value | | |
| | 15% ALA[a] | | | 30% ALA[a] | | | | Ratio | ALA | Ratio x ALA |
	L_a	M_b	H_c	L_a	M_b	H_c				
LAUR	0.2	0.6	0.8	0.2	0.3	0.6	0.01	***	***	NS
MYRIST	2.6	4.8	6.2	0.9	3.3	5.7	0.72	***	**	NS
PALM	46.4^{ab}	48.3^b	46.4^{ab}	41.0^a	43.3^a	48.1^b	12.41	*	**	*
PALMO	3.6^a	4.5^b	4.5^b	3.5^a	4.0^{ab}	6.2^c	0.68	***	NS	***
SA	17.5	16.2	16.0	17.0	13.9	15.5	7.19	NS	NS	NS
OA	67.9	65.4	63.8	65.6	58.2	65.3	69.40	NS	NS	NS
LA	47.2	43.3	37.7	38.8	35.4	30.0	10.48	***	***	NS
GLA	0.3	0.3	0.3	0.2	0.2	0.2	0.00	NS	***	NS
AA	3.1	2.8	2.6	2.1	2.0	1.6	0.09	***	***	NS
ALA	8.0^a	8.7^a	8.6^a	14.2^b	15.0^b	17.9^c	2.27	**	***	**
EPA	0.2^a	0.2^a	0.2^a	0.3^{ab}	0.4^b	0.5^c	0.00	***	***	**
DPA	0.4	0.4	0.6	0.5	0.6	0.6	0.02	**	*	NS
DHA	2.9	3.1	3.5	2.7	3.1	3.2	0.19	**	NS	NS
TOTAL n-6	50.5	46.4	40.6	41.2	37.6	31.8	12.10	***	***	NS
TOTAL n-3	11.3	12.4	12.9	17.6	19.0	22.2	3.16	***	***	NS

L_a, low ratio of saturated fatty acids: linoleic and oleic acids; M_b, medium ratio of saturated fatty acids: linoleic and oleic acids; H_c, high ratio of saturated fatty acids: linoleic and oleic acids.
[a]Expressed as a percentage of total fatty acids.

Data within a row with different superscripts are significantly different.
(NS $p \geq 0.05$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

LAUR= C12:0; MYRIST= C14:0; PALM= C16:0; PALMO= C16:1; SA= C18:0; OA= C18:1n9; LA= C18:2n6; GLA= C18:3n6; AA= C20:4n6; ALA= C18:3n3; EPA= C20:5n3; DPA= C22:5n3; DHA= C22:6n3; TOTAL n-6= C18:2n6 + C18:3n6 + C20:4n6; TOTAL n-3= C18:3n3 + C20:5n3 + C22:5n3 + C22:6n3.

SFA were the main focus of diets in Study 2. As such, we incorporated higher levels of SFA into the feed rather than manipulating the n-6 and n-9 FA content. We used coconut oil in Study 2 to serve this function because it is a vegetable source of SFA compared to the use of lard

in Study 1. For years, concern over the possible detrimental health effects of coconut oil revolved around its high SFA content (~90%, expressed as a percentage of total FA) and therefore its subsequent atherogenic effect (Marina, Che Man, Amin 2009). However, many studies have demonstrated improved lipid profiles, with lowered TC, lipoproteins and PL with coconut oil supplementation (Nevin and Rajamohan, 2004). The reason being that coconut oil is largely composed of medium chain triglycerides (MCTs) (~65% of total FA in coconut oil), which contain SFA with carbon chain lengths of 6–12 atoms (Marten, Pfeuffer, Schrezenmeir 2006). MCTs are rapidly absorbed and metabolized in the liver into energy and unlike LCPUFA, they do not participate in cholesterol biosynthesis and transport (Dayrit 2003; Marina, Che Man, Amin 2009). However, lipid metabolism is quite different in laying hens compared to humans. Hens have a poorly developed lymphatic system, therefore lipoproteins are secreted directly into the portal system (Hermier 1997). Laying hens are able to synthesize their own TG, cholesterol and PL through *de novo* hepatic lipogenesis (Hermier 1997).

One of the potential concerns with supplementing the layer diet with high amounts of SFA was the accumulation of even higher amounts in the egg yolk as compared to conventional eggs. We not only observed a significant increase in yolk SFA but also an interaction between ALA and SFA:LA+OA ratio. Therefore, the benefits of improved FA profile in terms of increasing n-3 PUFA conversion must be weighed against the potential downsides of increased SFA deposition, albeit the increases observed were modest and may not result in a major biological change. Additionally, increasing the SFA:LA+OA ratio led to a substantial decrease in n-6 PUFA, and modest increases in the LAUR content (due to the coconut oil). Human trials are necessary to determine the potential effects of these eggs on indices of health. However, many studies have shown that there are substantial differences among SFA in terms of their

cholesterol-raising effects. Investigators are in agreement that the total high density lipoprotein (HDL) ratio is more sensitive and specific than is TC as an indicator of atherogenic risk (Stampfer and others 1991; Kinosian and others 1995; Assmann and others 1996; Mensink and others 2003). A meta-analysis by Mensink and others (2003) found that despite LAUR being the most potent total and LDL cholesterol–raising SFA, it had the most favorable effect on the total: HDL ratio in human subjects than any other fatty acid, either saturated or unsaturated. After LAUR, the total: HDL ratio was more favorably affected by SA, MYRIST and PALM, in order from most to least favorable.

Our findings from Study 2 are in agreement with Wignjosoesastro, Brooks and Harrick (1972), where they found an addition of 10% coconut oil in the layer diet led to increased LAUR and MYRIST and decreased SA, OA and LA in the yolk fat fraction. The only difference was that our diets were not able to decrease SA and OA due to the significantly lower level of coconut oil inclusion (the highest level of inclusion in our diets was 4.16% in the H1 group) in combination with flax and corn oils. In fact, dietary treatments had no effect on the content of SA and OA in the egg yolk.

Although LAUR is an MCT found in large quantities in coconut oil (~48%), only minor amounts were detected in the yolk, whereas, MYRIST (a common SFA found in ~19% in coconut oil) resulted in much larger quantities in the yolk compared to LAUR. It is evident that the rapid utilization of MCTs for energy resulted in minor deposition into yolk. Human and animal studies have shown that MCTs are preferentially oxidized, in turn, leading to increased thermogenesis (Kasai and others 2002; Noguchi and others 2002; Hill and others 1989; Baba, Bracco, Hashim 1982). Furthermore, isotopic tracer and serum fatty acid analyses (Rodriguez and others 2003; Nagata and others 2003) suggest that MCTs might be able to spare oxidation of

PUFAs. This hypothesis could offer an explanation of the greater levels of yolk n-3 PUFAs in Study 2 eggs compared to Study 1 eggs, despite the same level of ALA in the diet (30% ALA). Further investigation using hens of the same age and strain is needed to fully elucidate this finding.

Excluding SA and OA, the level of ALA in the diet affected all other FA, with the exception of PALMO and DHA and the SFA:LA+OA ratio affected all other FA except GLA. Increasing dietary ALA resulted in marked decreases in the remaining FA, however significant increases were found in ALA, EPA, DPA and total n-3 PUFA. Total n-3 PUFA content was highest in the high ratio groups, with the higher ALA group containing the most (22.2mg total n-3 PUFA/g of yolk), however better LCPUFA deposition was found in the lower ALA group, which contained 3.5mg DHA/g of yolk. Increasing the dietary ALA content above 15% did not affect yolk DHA. In fact, slight decreases were observed in the 30% ALA group. This suggests that retro conversion of DHA back to DPA and EPA may have occurred, considering increases in these FA were observed in the 30% group, or that ALA conversion was suppressed, or both. Conversely, increasing the SFA:LA+OA ratio resulted in marked increases in the remaining FA, however significant decreases were found in LA, AA and total n-6 PUFA. In this case, DHA did increase with an increasing SFA:LA+OA ratio suggesting the LA:ALA ratio should not be the only consideration when attempting to enhance ALA conversion to LCPUFA. A significant interaction ($P<0.05$) between the ratio and ALA was observed in PALM, PALMO, ALA, and EPA, with total n-3 PUFA close to significance at $p=0.056$.

The n-6:n-3 PUFA ratio in the eggs from diets in Study 2 decreased with 1) increasing dietary SFA:LA+OA ratio and 2) increasing dietary ALA. According to Simopoulos (2002), humans evolved on a diet in which the ratio of n-6:n-3 PUFA was about 1, whereas the ratio in

Western diets is 15-17. Conventional eggs typically have an n-6:n-3 PUFA ratio even higher than the typical Western diet, at approximately 20. All of the eggs in this study were within the suggested optimal range of 1-4 for the ratio of n-6:n-3 PUFA, with the H2 group having the lowest ratio at 1.4.

5.4.3. Sensory analysis

Results from descriptive analysis on cooked eggs from Study 2 are shown in Table 5.6. A highly significant difference ($P<0.001$) was observed over the range of scores by individual panelists. Although training helps to decrease the panelist variability due to different sensitivities and use of the line scale, it cannot completely eliminate it (Lundahl and McDaniel 1988). Interaction effects were observed between panelist and replication for creamy and sweet aromas, and creamy, buttery, and sweet flavours. This interaction was investigated and no one particular panelist was responsible for the inconsistency. Creamy flavour was the only attribute that showed a significant panelist x treatment interaction. In all samples, the most intense aroma and flavour attribute was described as 'egg', which is in accord with our previous findings (Goldberg and others 2012).

Table 5.6: Descriptive analysis results from three-way analysis of variance (T=Dietary Treatment (n=6); P=Panelist (n=8); Replication (n=3)) and Fisher's least significant difference test for cooked eggs from hens consuming different ratios of saturated fat: linoleic and oleic acids and content of alpha-linolenic acid

				Dietary Treatment					
				Mean intensity ratings – 0 to 15 cm line scale (SEM)					
	Source of variation (F-value)			15% ALA[a]			30% ALA[a]		
	T	P	R	L_a	M_b	H_c	L_a	M_b	H_c
Aroma									
Egg	0.79	43.26	2.65	8.1	9.0	9.1	9.0	9.0	8.9
	NS	***	NS	(0.8)	(0.8)	(0.6)	(0.6)	(0.8)	(0.9)
Creamy	1.01	14.15	0.77	3.5	3.4	3.9	3.9	3.7	4.3
	NS	***	NS	(0.5)	(0.6)	(0.6)	(0.5)	(0.6)	(0.8)
Buttery	0.19	50.53	0.49	4.8	5.0	5.2	5.3	5.3	5.3
	NS	***	NS	(0.7)	(0.7)	(0.7)	(0.8)	(0.7)	(0.9)
Sweet	0.82	9.03	1.58	1.6	2.4	2.1	1.9	1.9	2.1
	NS	***	NS	(0.3)	(0.6)	(0.6)	(0.5)	(0.5)	(0.6)
Flavour									
Egg	1.50	21.74	1.07	9.3	8.4	9.8	9.1	10.1	8.9
	NS	***	NS	(0.7)	(0.8)	(0.6)	(0.7)	(0.7)	(0.8)
Creamy	1.35[b]	11.95[b]	0.08	4.7	3.1	3.4	3.4	3.4	4.0
	NS	***	NS	(0.7)	(0.6)	(0.4)	(0.6)	(0.5)	(0.6)
Buttery	1.33	13.38	0.21	5.6	4.3	4.5	4.4	4.4	5.1
	NS	***	NS	(0.8)	(0.7)	(0.5)	(0.7)	(0.6)	(0.8)
Sweet	1.07	34.14	0.64	1.6	1.9	1.7	1.8	1.4	1.6
	NS	***	NS	(0.3)	(0.4)	(0.4)	(0.4)	(0.3)	(0.3)

Interaction *F*-values and significance not shown in Table; PxT, Creamy flavour 1.65*; PxR, Creamy aroma 2.91***; Sweet aroma 5.65***; Creamy flavor 1.82*; Buttery flavour 1.88*; Sweet flavour 2.32**. ANOVA, analysis of variance; ALA, alpha-linolenic acid; L_a, low ratio of saturated fatty acids: linoleic and oleic acids; M_b, medium ratio of saturated fatty acids: linoleic and oleic acids; H_c, high ratio of saturated fatty acids: linoleic and oleic acids.
[a]Expressed as a percentage of total fatty acids.
[b]New *F*-value as determined by testing the main effects by the interaction effect.
Levels of significance: NS $P \geq 0.05$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

No significant differences in sensory attributes between dietary treatments for cooked egg samples were found. It is possible that the reason for this is that the level of vitamin E included in these diets was adequate to prevent oxidation that would result in aroma and flavour changes. Limited data are available on the sensory effects of eggs from hens supplied with high doses of vitamin E in their diet. However, Leeson, Caston and MacLaurin (1998) found that 100 IU compared to 10 IU vitamin E/kg of diet accentuated the effects of decreased acceptability in boiled eggs from hens fed 20% flaxseed, but not in hens fed a control diet without flaxseed. The authors suggested that the higher dose of vitamin E may have resulted in a pro-oxidant effect that led to decreased acceptability. In contrast, Franchini and others (2002) found that an even greater dose of 200 IU vitamin E/ kg of diet did not alter sensory perception compared to hens fed no additional vitamin E. However, both studies utilized an untrained consumer panel, whereas a trained descriptive panel was used to assess specific sensory attributes in this study. Also, differences in the cooking and sensory testing methods could have resulted in such differences to be significant. The degree of vitamin E supplementation in the current study was unchanged across dietary treatments. Because significant sensory differences in the resultant eggs were not observed, the possibility of this level of vitamin E causing sensory changes, particularity negative, is not reasonable. Our results in this regard align with our objective of observing non-significant aroma and flavour changes. Although improving the sensory profile of enriched eggs would be ideal, for instance, as an increase in egg flavour, this would be difficult to achieve.

In Figure 5.1, the graphical presentation of the correlation loadings using PLS depicts overall possible relationships between yolk FA and sensory attributes of cooked eggs. The first observation to note is the clear separation between dietary treatments. Based on the greater

separation between the ALA groups (n=2) compared to the SFA:LA+OA ratio groups (n=3), it seems that dietary ALA plays a larger role in the sensory outcomes. There also appears to be similar separations between the two L and two H treatments, however a much larger separation exists between the M treatments, suggesting a greater sensory difference between them. In terms of FA, there are three main clusters. The first cluster found closer to the low ALA groups consists of all n-6 PUFA, including LA, AA, and GLA. The second, which is closer to the high ratio groups is DHA, LAUR, MYRIST and PALMO. The third, which is found closer to the high ratio groups, are the n-3 PUFA, including ALA, EPA and DPA. The most interesting finding is that DHA is more related to SFA as opposed to the other n-3 PUFA. With regard to the association between FA and sensory attributes, there are four main observations. First, egg flavour appears to be closely related to the n-3 PUFA, a finding that was corroborated in our previous study analyzing the sensory differences from eggs procured from hens fed hemp (Goldberg and others, 2012). The difference in this study is that DHA was unrelated to the other n-3 PUFA, which was not the case in Goldberg and others (2012). Secondly, egg, creamy and buttery aromas are more related to n-3 PUFA, and third, sweet, buttery and creamy flavours are more related to the n-6 PUFA. Lastly, sweet aroma is more related to SFA and DHA. Based on these observations, there is a possibility that the n-3 PUFA and SFA are more closely related to aromas whereas n-6 PUFA to flavours.
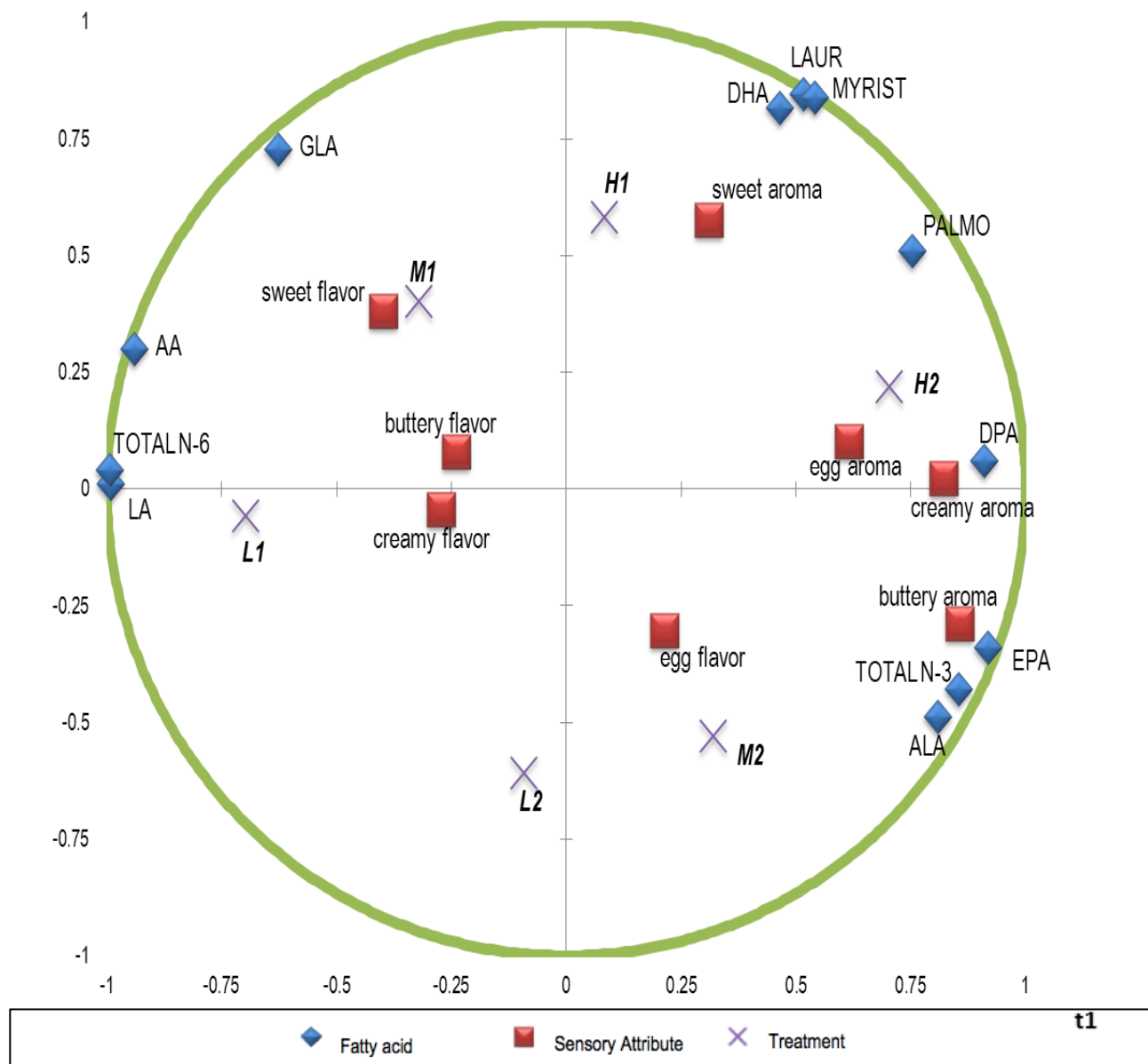
Figure 5.1: An overview of the correlation loadings from partial least squares analyses with yolk fatty acids as X-variables and sensory attributes of cooked egg samples as Y-variables from hens consuming different ratios of saturated fat: linoleic and oleic acids and content of alpha-linolenic acid

L= low; M= medium; H= high ratio of saturated fatty acids: linoleic and oleic acids
1= 15% ALA; 2= 30% ALA (expressed as a percentage of total fatty acids)
LAUR= C12:0; MYRIST= C14:0; PALMO= C16:1; LA= C18:2n6; GLA= C18:3n6; AA= C20:4n6; ALA= C18:3n3; EPA= C20:5n3; DPA= C22:5n3; DHA= C22:6n3; TOTAL n-6= C18:2n6 + C18:3n6 + C20:4n6; TOTAL n-3= C18:3n3 + C20:5n3 + C22:5n3 + C22:6n3.

5.5. Conclusions

The present research objectives were to assess the impact of altering the LA content and the SFA:LA+OA ratio in laying hen diets on the conversion of n-3 PUFA into the resultant eggs. For the latter study, there was an additional objective of examining the potential for changes in the sensory attributes of the cooked eggs due to a greater change in yolk fatty acids. These objectives were addressed by testing egg yolks for fatty acid content and by utilizing a trained sensory panel to determine the aroma and flavour attribute intensities of cooked eggs.

The amount of ALA and LA in the diet and the dietary ratio of SFA:LA+OA play a major role in the fatty acid composition of resultant eggs. The most important observations to note from the current research are 1) that increasing the dietary LA content led to a decrease in EPA and an increase in DPA and 2) that the SFA:LA+OA ratio plays more of a role in ALA conversion to LCPUFA, especially with regard to DHA, compared to increasing ALA content alone. Despite differences in the fatty acid composition of the eggs, there were no sensory differences in cooked eggs across all dietary treatments. However, egg flavour appeared to be positively correlated with n-3 PUFA, with the exception of DHA. This suggests that there may be potential to alter the sensory profile in cooked eggs by altering the SFA:LA+OA ratio in the diet. It also suggests that the addition of pre-formed DHA in the laying hen diet may not impact egg flavour, but this does not rule out the possibility that other aromas or flavours may be developed. Further research is needed to understand the close association between egg flavour and n-3 PUFA and before this can be achieved, it is essential that egg flavour be clearly defined in terms of the mixture of volatiles that compose it.

In summary, the differences in LA in Study 1 diets were not large enough to result in any major impact on ALA conversion. Results from Study 2 show that it is possible to incorporate

modest amounts of LAUR into egg yolk through coconut oil feeding and that a higher SFA:LA+OA ratio modestly increases the conversion of ALA into LCPUFA without negatively affecting the sensory quality in cooked table eggs. Based on this research, a high SFA:LA+OA ratio (using coconut oil as the SFA source) coupled with a low n-6:n-3 PUFA ratio in layer diets is recommended to enhance ALA conversion, MCT deposition, and decrease n-6 PUFA in the egg yolk.

5.6. Acknowledgment

CHAPTER 6: INTERACTIONS BETWEEN CANOLA MEAL AND FLAXSEED OIL IN THE DIETS OF WHITE LOHMANN HENS ON FATTY ACID PROFILE AND SENSORY CHARACTERISTICS OF RESULTANT EGGS

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6.1. Abstract

The current study was designed to assess the fatty acid composition and sensory attributes of eggs procured from hens consuming diets containing CM and/or FO. Ninety-six group-caged White Lohmann hens received 1 of 4 isonitrogenous and isoenergetic diets for a period of 4 weeks. Diets were arranged in a 2 x 2 factorial design, containing 24% CM, 7.5% FO, both or neither (control). All yolk FA were affected by FO inclusion, with the exception of SA and DPA. Only SA was affected by CM inclusion. Additionally, significant interactions between CM and FO were observed for LA and total n-6 PUFA, with DPA approaching significance (p=0.069). Trained panelists (n=8) evaluated 7 aroma ('egg', 'creamy', 'buttery', 'salty', 'sweet', 'barny' and 'oceanic') and 6 flavour ('egg', 'creamy', 'buttery', 'salty', 'brothy' and 'oceanic') attributes of cooked egg product. No significant differences (P>0.05) in aroma attributes were found between eggs from different dietary treatments. However, egg, creamy, buttery and oceanic flavours were significantly different between the dietary treatments (P<0.05). While oceanic flavour significantly increased with inclusion of FO, egg and creamy flavours showed a significant decrease (P<0.05). Although CM addition alone did not result in significant sensory changes, the pairing of CM and FO resulted in even greater sensory changes than using FO alone, specifically with regard to egg flavour. According to results from partial least squares analyses, there appears to be a strong association between oceanic flavour and n-3 PUFA. Oppositely, egg, creamy and buttery flavours are more correlated with the presence of n-6 PUFA and PALM. This experiment provides evidence that the interaction between CM and FO in the White Lohmann hen diet results in sensory changes of cooked eggs associated in part with changes in yolk fatty acid content.

6.2. Introduction

With the health benefits of n-3 PUFA well established, growing concern over the lack of consumption of such critical FA is evident. Dietary Reference Intake values for ALA range from 1.1-1.6 g/day for females and males, respectively (Health Canada 2012c). The American Dietetic Association and Dietitians of Canada recommend the consumption of 500 mg/day of combined EPA and DHA for the primary prevention of coronary diseases (Kris-Etherton, Grieger, Etherton 2009; Kris-Etherton and others 2007). The production of n-3 enriched eggs is a viable strategy to obtain critical FA for optimal health, especially in westernized diets where there has been a shift towards a greater n-6 PUFA intake, leading to more inflammation (Blasbalg and others 2011). Flaxseed, a commonly used ingredient in hen diets, provides a convenient package for high-quality oil rich in ALA at approximately 23% in its whole form and 53% in the oil fraction, that is necessary for egg enrichment (Jia and others 2008; Leeson and Summers 2005). In fact, increasing the amount of flaxseed in the laying hen diet by 1% would result in an increase in n-3 fatty acid deposition by 40 mg per egg (Leeson and Summers 2005). However, too much dietary flax can lead to off-aroma and flavours in the resultant eggs (Caston, Squires, Leeson 1994; Hayat and others 2010a; Leeson, Caston, MacLaurin 1998; Scheideler, Froning, Cuppett 1997).

An additional challenge is the potential for other feed ingredients to interact negatively with the source of n-3 PUFA. One such ingredient is CM, a financially attractive and rich source of protein that remains after the solvent extraction of canola oil. It was discovered in the 1970's that some layer strains such as Rhode Island Red (RIR) laid tainted eggs when fed rapeseed or CM, therefore it was hypothesized early on that the taint could be the result of brown shell colour. However, it was demonstrated that Brown Leghorns whose eggs have white shells could also produce tainted eggs when fed high levels of rapeseed meal, and alternatively, the taint was

not found in eggs from a New Hampshire Red hybrid, which lay brown eggs (Bolton, Carter, Jones 1976). An autosomal semi-dominant mutant gene with variable expression dependent on environmental factors was determined to be responsible. It is this gene that affected the capacity of flavin-containing monooxygenase isoform 3, FMO3, to oxidise TMA into the odourless TMA oxide (TMAO). The unfortunate side effect of the TMA accumulation in chicken blood was that TMA would subsequently accumulate in the egg yolk. Trimethylamine may be produced from certain precursors, either TMAO, sinapine (ester of choline and sinapic acid), lecithin or directly from choline by the action of microorganisms in the lower gut (Goh and others 1979; Jeroch and others 1999; Pearson and others 1983b). Additionally, goitrin, an antinutritional factor formed by the action of myrosinase on glucosinolates, can inhibit TMA oxidation by competing for the active site of FMO3, leading to even more TMA accumulation in egg yolk (Pearson and others 1983a).

Due to past problems with fishy taint, many producers limited their use of CM in hen diets but throughout the years the egg industry has selectively bred hens without this mutation to eliminate this problem. Despite their efforts, there is still potential for high levels of CM to interact with n-3 PUFA in the hen diet, which may negatively affect the sensory properties of the eggs. This of course, may be more problematic in functional feeds whereby yolk n-3 enrichment is the goal. It is evident that both ingredients, flaxseed and CM, have demonstrated sensory concerns when used in isolation. To date, there is no available literature on the potential for interactions between FO and CM in laying hen diets in regards to sensory quality. Therefore the objective of the current study is to assess the potential for such interactions in White Lohmann layer diets and to investigate possible relationships between sensory attributes and specific yolk FA. Additionally, fishy taint has not been well described in the literature. In using a trained

sensory panel in this research, a specific set of attributes will be developed that will help further describe this "fishy" phenomenon.

6.3. Materials and Methods

6.3.1. Bird housing and environment

Ninety-six White Lohmann hens at 60 weeks of age (Clark Hy-Line Inc, Brandon, MB, Canada) were used in the current study. Hens were caged in groups of three (468cm² floor space allowance), under semi-controlled environmental conditions (temperature was controlled via supplemental heat and/or ventilation) including exposure to a 16-hour photoperiod. Feed and water were provided for *ad libitum* consumption. Feeding troughs were divided to ensure hens could only access their particular dietary treatment and they were also fitted with weighted screens that, when placed on top of feed, minimized feed wastage. All of the procedures used during this study were approved by The University of Manitoba's Animal Care Protocol Review Committee, in accordance with recommendations established by the Canadian Council on Animal Care (Canadian Council on Animal Care 1993).

6.3.2. Diets

The diets used in the current study are shown in Table 6.1. Diets were isoenergetic and isonitrogenous, and formulated to meet the requirements of laying hens consuming 100 g of feed per day (National Research Council 1994). All diets were predominantly wheat-based, using limestone as the calcium source. Diets only differed in the oil type (corn vs. flaxseed) and meal type (canola vs. soybean) used. Low energy CM (Bunge, Altona, MB, Canada) was used in order to match the energy profile with soybean meal as closely as possible.

Table 6.1: Ingredient composition of layer diets containing canola meal, flaxseed oil or both

Ingredients (%)	Control	Canola Meal	Flax Oil	Canola & Flax
Ground canola meal	0.00	23.63	0.00	23.63
Ground soybean meal	17.60	0.00	17.60	0.00
Wheat	59.85	58.11	59.85	58.11
VM Premix[1]	2.50	2.50	2.50	2.50
Salt	0.30	0.30	0.30	0.30
Limestone	10.19	10.02	10.19	10.02
Dicalcium phosphate	1.68	1.40	1.68	1.40
Lysine	0.21	0.32	0.21	0.32
DL-Methionine	0.16	0.10	0.16	0.10
Threonine	0.01	0.00	0.01	0.00
Corn oil	7.50	7.50	0.00	0.00
Flaxseed oil	0.00	0.00	7.50	7.50
Calculated Composition				
AMEn (Poultry; Kcal/kg)	3095.00	3121.06	3095.00	3121.06
Crude Fat (%)	9.24	9.66	9.24	9.66
Crude Protein (%)	18.50	18.50	18.50	18.50
Total Lysine (%)	0.98	1.00	0.98	1.00
Calcium (%)	4.10	4.10	4.10	4.10
Total Phosphorus (%)	0.67	0.77	0.67	0.77
Available Phosphorus (%)	0.45	0.45	0.45	0.45
Sodium (%)	0.15	0.15	0.15	0.15
Chloride (%)	0.22	0.22	0.22	0.22
Methionine (%)	0.40	0.40	0.40	0.40
Threonine (%)	0.60	0.60	0.60	0.60

[1]Provided per kg of diet: 11,000 IU vitamin A, 3000 IU vitamin D3, 150 IU vitamin E, 3 mg of vitamin K(as menadione), 0.02 mg cyanocobalamin, 6.5 mg riboflavin, 4 mg folic acid, 10 mg of calcium pantothenate, 40.1 mg niacin, 0.2 mg biotin, 2.2 mg thiamine, 4.5 mg pyridoxine, 1000 mg choline, 125 mg of ethoxyquin (antioxidant), 66 mg Mn (as manganese dioxide), 70 mg Zn (as zinc oxide), 80 mg Fe (ferrous sulphate), 10 mg Cu (as copper sulphate), 0.3 mg Se (as sodium selenite), 0.4 mg I (as calcium iodate), and 0.67 mg iodized salt.

VM= Vitamin-Mineral; AMEn= Nitrogen corrected apparent metabolisable energy

To avoid the possibility of interactions due to other components in flaxseed, such as fiber and lignans, FO was used as a source of FA. The purpose of choosing corn oil for the control was because of its extremely low n-3 PUFA content. Diets containing FO were designed to

contain 4% ALA (expressed as a percentage of total FA) in order to produce eggs with an n-3 nutrient content claim in Canada, which is at least 300 mg n-3 PUFA per egg (Health Canada 2012b). Because of the higher fat content of the diets (9-10%), additional alpha-tocopherol (vitamin E) was added to all diets at 150 international units (IU) per kilogram of feed as part of the 2.5% vitamin-mineral premix.

6.3.3. Dietary analysis

Feed samples were analyzed for dry matter, crude protein and crude fat according to established procedures (AOAC International 1995). The fatty acid composition of the test diets was determined using standard gas chromatographic techniques of the fatty acid methyl esters (AOAC International 1990), using C17:1 methyl ester as an internal standard.

6.3.4. Experimental protocol

Hens were weighed and caged in groups of 3 in rollout style cages, and allowed an adaptation period of 7 days before being randomly assigned to receive 1 of 4 dietary treatments. The study was 4 weeks in duration. Body weights of hens were recorded at the start of the trial, and at weekly intervals. Feed consumption was determined for the entire week and average daily feed intake and feed efficiency were calculated. Three eggs from each cage were weighed daily and the average weight was recorded daily and an average egg production rate was calculated. Prior to sensory analysis, eggs were stored in trays in the dark at 4ºC overnight.

6.3.5. Fatty acid analysis

At week 4, one egg from each cage was chosen for fatty acid analysis from the current study. Egg yolks were separated from the albumen and stored in plastic bags at -80°C until analysis. Fatty acids were extracted from the egg yolk according to the methods of Folch, Lees

and Stanley (1957). The fatty acid composition of the yolks was determined in the same manner as the feed.

6.3.6. Sample preparation

Four randomly chosen eggs from week 4 of the experiment of similar weight (56.0±2.0g) from each treatment were pooled and cooked according to the methods of Goldberg and others (2012). To summarize, eggs were homogenized and then stovetop cooked in covered glass jars for approximately 15 min. All prepared samples were analyzed within 10 min after preparation was complete and the jars were kept at a constant temperature of 55°C in a heated water bath until panelists evaluated the samples.

6.3.7. Recruitment

Eight panelists (6 females, 2 males, aged between 20 and 55) who were students and staff of The University of Manitoba were recruited (See Appendices G-I) to participate in the sensory evaluation. The sensory analysis component of the research received ethical approval from The University of Manitoba's research ethics board (See Appendices C-F). The criteria for participation included open availability, an interest in the panel, and no aversion or allergies to eggs and any ingredients present in any other products to be used for training purposes. Panelists completed a questionnaire (See Appendix K) to ensure that no allergies were present, and written consent (See Appendix J) was obtained.

6.3.8. Descriptive analysis

Descriptive analysis was conducted using a modified method by Stone and Sidel (2004). Prior to analysis all panelists participated in 6 training sessions of 45 min in duration at which

time egg samples were presented coded with a randomly selected 3-digit number. During each

training session, panelists evaluated the aroma and flavour (See Appendices L-N) of each of the

randomized samples and developed an agreed vocabulary of attributes. The final agreed

descriptive vocabulary consisted of 7 aroma and 6 flavour attributes (Table 6.2).

Table 6.2: Aroma and flavour definitions and standard products used in sensory training sessions for cooked eggs procured from hens consuming canola meal, flaxseed oil or both

Attribute	Definition	Standard Amount[1]/Amount
Aroma		
Egg	Aroma associated with whole egg	blended commercial egg cooked and presented as for experimental samples (Superstore, Grade A large, Winnipeg MB)/15g
Creamy	Aroma associated with whipping cream	half whipping cream (Canada Safeway, Lucerne Brand, Winnipeg, MB), half filtered water/10g
Buttery	Aroma associated with unsalted butter	unsalted butter (Superstore, Foremost Dairies, Winnipeg, MB)/0.5g
Salty	Aroma associated with chicken broth	boiled chicken broth/10g
Sweet	Aroma associated with 2% milk	2% milk (Superstore, Beatrice Brand, Winnipeg, MB)/10g
Barny	Aroma associated with straw	straw/0.1g
Oceanic	Aroma associated with kelp	Half of kelp tablet (Jamieson brand, 650 mcg iodine)/0.5g
Flavour/Taste		
Egg	Flavour associated with whole egg	blended commercial egg cooked and presented as for experimental samples (Superstore, Grade A large, Winnipeg MB)/15g
Creamy	Flavour associated with whipping cream	whipping cream (Superstore, Foremost Dairies, Winnipeg, MB)/5g
Buttery	Flavour associated with unsalted butter	unsalted butter (Superstore, Foremost Dairies, Winnipeg, MB)/5g
Salty	Taste associated with salt solution	0.25% w/v coarse salt solution (Sifto Canada Inc., Mississauga ON) dissolved in filtered water/5g
Brothy	Flavour associated with chicken broth	boiled chicken broth[2]/5g
Oceanic	Flavour associated with oceanic aroma	Half of kelp tablet (Jamieson brand, 650 mcg iodine)/0.5g

[1]Placed in 60mL plastic portion cup and capped with plastic lid about 1 hour prior to evaluation served at room temperature; except for butter samples at 4°C.

[2]liquid from 260g of boneless skinless chicken breast (Canada Safeway, Granny's Brand, Winnipeg MB) placed in 2L of water brought to a boil and simmered for 27 minutes when the chicken had reached an internal temperature of 75°C.

Other products, including cooked commercial eggs, dairy products, chicken broth, straw, kelp tablets and saline solution were also evaluated and used as reference points for each attribute.

On the days of sensory analysis, samples were prepared as described above (Section 2.6). All sensory evaluations were conducted in individual partitioned workstations at the sensory laboratory at The University of Manitoba. Workstations were equipped with SIMS 2000 (2010) computerized sensory software (Sensory Integrated Management System, Morristown, NJ, U.S.A.). Light from incandescent bulbs directed through red opaque plastic was used in workstations to mask any potential colour differences between samples. Samples were coded with randomly selected 3-digit numbers and order of tasting between and within days was balanced to account for first order and carry-over effects. The aroma and flavour attributes of different samples were scored on unstructured 15cm line scales from 0 (low) to 15 (high). Each panelist was provided with filtered, room temperature water to cleanse his or her palate between tastings. Panelists' evaluated aroma attributes first, followed by flavour attributes (See Appendix O). Egg evaluations from each of the 4 dietary treatments were replicated 3 times on 3 separate days within the same week. At each of the 3 egg tasting sessions, panelists assessed 4 samples.

6.3.9. Statistical analysis

The current study was designed to be completely randomized, and all analyses were conducted using IBM SPSS Statistics (version 20.0). Fatty acids were analyzed using two-way ANOVA. Sensory data were analyzed using three-way ANOVA. The model included *Dietary Treatments (T)* as a fixed effect, and *Panelists (P)* and *Replications (R)* as random effects, and

two-way interactions of *Panelist by Dietary Treatment, Dietary Treatment by Replication* and *Panelist by Replication*. When interactions were not significant the sums of squares were pooled with the error (O'Mahony 1986). F values were recalculated with the additional sums of squares for error and the corresponding degrees of freedom. If a significant panelist-by-treatment interaction was observed, the main effects of panelist and treatment were tested by the interaction effect (See Appendix P) (Stone, Bleibaum, Thomas 2012). Fisher's LSD test was used to determine mean treatment differences when significant ($P<0.05$). A bi-plot was generated using PLS (XLSTAT version 2012) analysis using average values for all of the attributes to provide a visual perspective of the correlation between the samples in relation to the corresponding yolk FA and sensory attribute intensities.

6.4. Results and Discussion

6.4.1. Bird health

Mean values for egg production, egg weight, bird weight, feed consumption and feed conversion efficiency across treatments were not significantly different ($P≥0.05$) from one another. The following data are expressed as mean values ± the standard error of the mean (SEM). Egg production was $6.5 ± 0.11$ eggs per week, eggs weighed $65.2 ± 0.40$ g, average feed consumption was $109.0 ± 1.46$ g/day and feed conversion efficiency was $1.7 ± 0.03$ g of feed/g of egg. Birds initially weighed $1.9 ± 0.02$ kg, and by the end of the trial they weighed $2.0 ± 0.01$ kg. These results are consistent with other findings where it was demonstrated that as much as 25% of the layer diet could consist of CM, with no difference in feed intake, weight gain or feed efficiency compared to an equivalent amount of soybean meal in the diet (Leeson, Atteh, Summers 1987). The inherent astringency, and bitter and sour taste of sinapine has been held responsible for the decreased palatability of rapeseed meal in rodent and swine diets (Maga and

Lorenz 1973; Sosulski 1979), but levels used in this study did not impact feed consumption.

Qiao and Classen (2003) found that the levels of sinapine found in CM were not sufficient

enough to reduce feed intake in broiler chickens. Furthermore, Leeson, Atteh and Summers

(1987) also found that CM inclusion did not affect protein, fat, calcium, mineral retention and

energy utilization. Despite these findings, CM use is still restricted to less than full replacement

of soybean meal due to the low available energy content, high fiber, and presence of anti-

nutritional factors (Khajali and Slominski 2012; Mawson and others 1993). Of course, longer-

term studies may be needed to support these findings.

6.4.2. Fatty acid composition of egg yolks

Results from yolk fatty acid analysis are shown in Table 6.3.

Table 6.3: Fatty acid composition of egg yolks collected from hens consuming canola meal, flaxseed oil or both

Fatty Acid (mg/g yolk)	Treatment				SEM	P-Value		
	Control	Canola	Flax	Canola + Flax		Flax	Canola	Canola x Flax
PALM	50.2	49.4	44.7	43.3	17.95	***	NS	NS
PALMO	2.8	2.6	4.4	4.0	0.28	***	NS	NS
SA	20.6	18.2	20.5	20.3	3.09	NS	*	NS
OA	72.1	72.2	82.2	83.8	31.99	***	NS	NS
LA	51.5[b]	55.8[c]	27.9[a]	26.4[a]	10.24	***	NS	*
GLA	0.3	0.4	0.1	0.1	0.00	***	NS	NS
AA	4.6	4.5	1.7	1.5	0.09	***	NS	NS
ALA	0.9	1.0	20.5	21.2	3.90	***	NS	NS
EPA	0.0	0.0	0.6	0.6	0.01	***	NS	NS
DPA	0.6	0.5	0.5	1.4	0.55	NS	NS	NS
DHA	1.2	1.3	2.9	3.0	0.14	***	NS	NS
TOTAL N-6	56.5[b]	60.6[c]	29.7[a]	28.1[a]	10.92	***	NS	*
TOTAL N-3	2.1	2.4	24.5	25.2	3.31	***	NS	NS

Levels of significance: NS p≥0.05; *P<0.05, **P<0.01, ***P<0.001.

Data within a row with different superscripts are significantly different.

DPA was the only FA unaffected by dietary treatment, although DPA was close to significance (p=0.069). All other FA were affected by FO inclusion with the exception of SA, which was only affected by CM. A significant interaction between CM and FO was found for LA and total n-6 PUFA. Both flax treatments did not differ in the amount of LA or total n-6 deposited in the yolk, however control yolks had significantly less of these two components compared to the CM group. In a different study, feeding hens 6% FO in the diet resulted in a deposition of 24 mg total n-3 PUFA / g yolk (Antruejo and others 2011). Our yolks yielded only slightly higher amounts (24.5 mg/g in flax treatment, and 25.2 mg/g in canola/flax treatment) despite 7.5% FO inclusion in the diets. This suggests that maximal deposition has been achieved around this level using vegetarian sources of n-3 PUFA, whereby further inclusion would not only result in impaired sensory quality, but would also be too expensive to be realistically used in industry.

6.4.3. Sensory analysis

Aroma and flavour definitions and standards used during training are shown in Table 6.2 and the results from sensory analysis in Table 6.4. There was a highly significant difference observed over the range of scores by individual panelists for all sensory attributes (P<0.001). This level of significance is typical in sensory studies because individuals may have their own frame of reference, despite the attempt to decrease this gap during training sessions. Also, variability in the ability of individuals to detect certain attributes has been noted, in particular, fishy notes (Griffiths, Land, Hobson-Frohock 1979). There were differences seen for replication for egg, creamy, buttery and salty aromas, suggesting that perhaps these attributes were more difficult for panelists to detect in a consistent manner over different sessions. This finding was also observed in previous sensory panels we have conducted (Goldberg and others 2012).

Table 6.4: Descriptive analysis results from three-way ANOVA (T=Dietary Treatment (n=4); P=Panelist (n=8); Replication (n=3)).

	Source of variation (F-value)			**Treatment (mean intensity ratings – 0 to 15 cm line scale (SEM))**			
	T	**P**	**R**	Control	Canola Meal	Flax Oil	Canola & Flax
Aroma							
Egg	0.76 NS	14.54 ***	3.68 *	7.5 (0.8)	8.6 (0.8)	8.0 (0.9)	7.7 (0.8)
Creamy	1.58 NS	15.13 ***	7.36 **	7.0 (0.8)	6.3 (0.8)	6.5 (0.8)	5.4 (0.8)
Buttery	0.24 NS	20.72 ***	4.81 *	5.3 (0.6)	5.2 (0.7)	5.1 (0.7)	4.9 (0.6)
Salty	0.03 NS	16.54 ***	3.40 *	4.7 (0.5)	4.8 (0.6)	4.8 (0.5)	4.9 (0.5)
Sweet	0.91 NS	10.49 ***	1.02 NS	3.1 (0.4)	3.1 (0.5)	3.4 (0.4)	2.6 (0.4)
Barny	1.48[b] NS	6.88[b] ***	1.34 NS	1.2 (0.3)	1.1 (0.2)	1.0 (0.2)	1.5 (0.2)
Oceanic	1.95[b] NS	9.02[b] ***	2.05 NS	1.3 (0.2)	1.8 (0.3)	1.6 (0.3)	2.0 (0.3)
Flavour							
Egg	3.11 *	14.24 ***	0.05 NS	7.7[b] (0.6)	7.5[b] (0.6)	6.4[b] (0.7)	5.8[a] (0.7)
Creamy	7.40[b] ***	17.73[b] ***	3.16 *	6.7[b] (0.6)	6.9[b] (0.6)	5.4[a] (0.7)	4.7[a] (0.6)
Buttery	3.70 *	12.50 ***	0.65 NS	5.8[b] (0.5)	5.2[b] (0.6)	4.6[ab] (0.6)	4.0[a] (0.5)
Salty	0.94 NS	9.11 ***	0.41 NS	3.2 (0.4)	2.7 (0.4)	3.2 (0.3)	3.2 (0.3)
Brothy	1.23 NS	10.46 ***	0.46 NS	2.8 (0.5)	2.2 (0.4)	3.1 (0.5)	2.8 (0.4)
Oceanic	2.90 *	3.95 ***	0.13 NS	0.9[a] (0.3)	0.9[a] (0.2)	1.7[b] (0.4)	1.8[b] (0.4)

Interaction F-values and significance not shown in Table; PxT, Barny aroma 1.59 NS; Oceanic

aroma 1.94*; Creamy flavour 2.25**; TxR, Brothy flavour 2.31*; PxR, Salty flavour 2.40**. ANOVA, analysis of variance;
[b]New F-value as determined by testing the main effects by the interaction effect.
Data within a row with different superscripts are significantly different.
Levels of significance: NS $P \geq 0.05$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Panelist x treatment interaction effects were observed for oceanic aroma and creamy flavour. There was also a significant treatment x panelist interaction for brothy flavour, and a panelist x replication interaction for salty flavour. These interactions were investigated and not one particular panelist was responsible for the inconsistency. In all samples, the most intense attribute was described as 'egg' aroma and flavour, and the least intense attributes were 'barny' aroma and 'oceanic' flavour.

Typically the presence of TMA results in 'egg taint' that is usually defined as a fishy aroma. However, in this study, treatment diets had no effect on the aroma attributes of the cooked eggs suggesting that TMA levels may not be affected by these diets. Of course, TMA analysis is needed in order to confirm this. Salty and brothy flavours were also unaffected by dietary treatment. Significant differences in egg, creamy and buttery flavour attributes between treatments were noted. There were also significant differences found for oceanic flavour, described by the panel as not fish specifically but more akin to dried kelp seaweed. Moreover, egg flavour intensity was decreased when CM and FO were paired, whereas oceanic flavour was not increased beyond what was contributed by the FO. This suggests that CM addition plays a minor role in enhancing oceanic flavour in n-3 enriched eggs. This is very promising, considering it has been found that as little as 5% dietary fish meal in broiler diets produce off-flavours in meat when combined with 20% rapeseed meal, but not when chickens were fed rapeseed alone (Hawrysh and others 1980). Sensory attributes not typically associated with egg

such as those described as being oceanic and barny are likely to result in decreased acceptance, therefore a consumer panel would be required to elucidate this hypothesis.

The graphical presentation of the correlation loadings using PLS (Figure 6.1) depicts overall possible relationships between cooked eggs from different dietary treatments, including specific yolk FA, and the sensory attributes. The first observation in analyzing this figure is the distinct separation of each dietary treatment, one in each of the four quadrants, suggesting large differences between them. The control group appears to be more related to sweet, creamy and buttery aromas in addition to egg, creamy and buttery flavours, all of which can be considered desirable attributes. The canola group was more associated with egg aroma. The flax group was more associated with salty and brothy flavours, whereas the canola/flax group was more related to oceanic, salty and barny aromas. The close association between oceanic flavour and both the canola/flax and flax groups is understandable considering the amount of FO, and thus n-3 PUFA are comparable between these diets. However, this particular attribute was closer to the canola/flax group suggesting an even stronger relationship; a possible additive effect.

In terms of FA, the control and canola groups were more related to total n-6 PUFA, including LA, GLA, AA as well as the saturate, PALM. However, the total n-3 PUFA, EPA and PALMO were closely related to both flax groups. Moreover, DPA was more related to canola/flax versus flax alone, and SA more related to flax versus canola/flax. Additionally, OA was more related to the flax groups versus the canola and control groups.
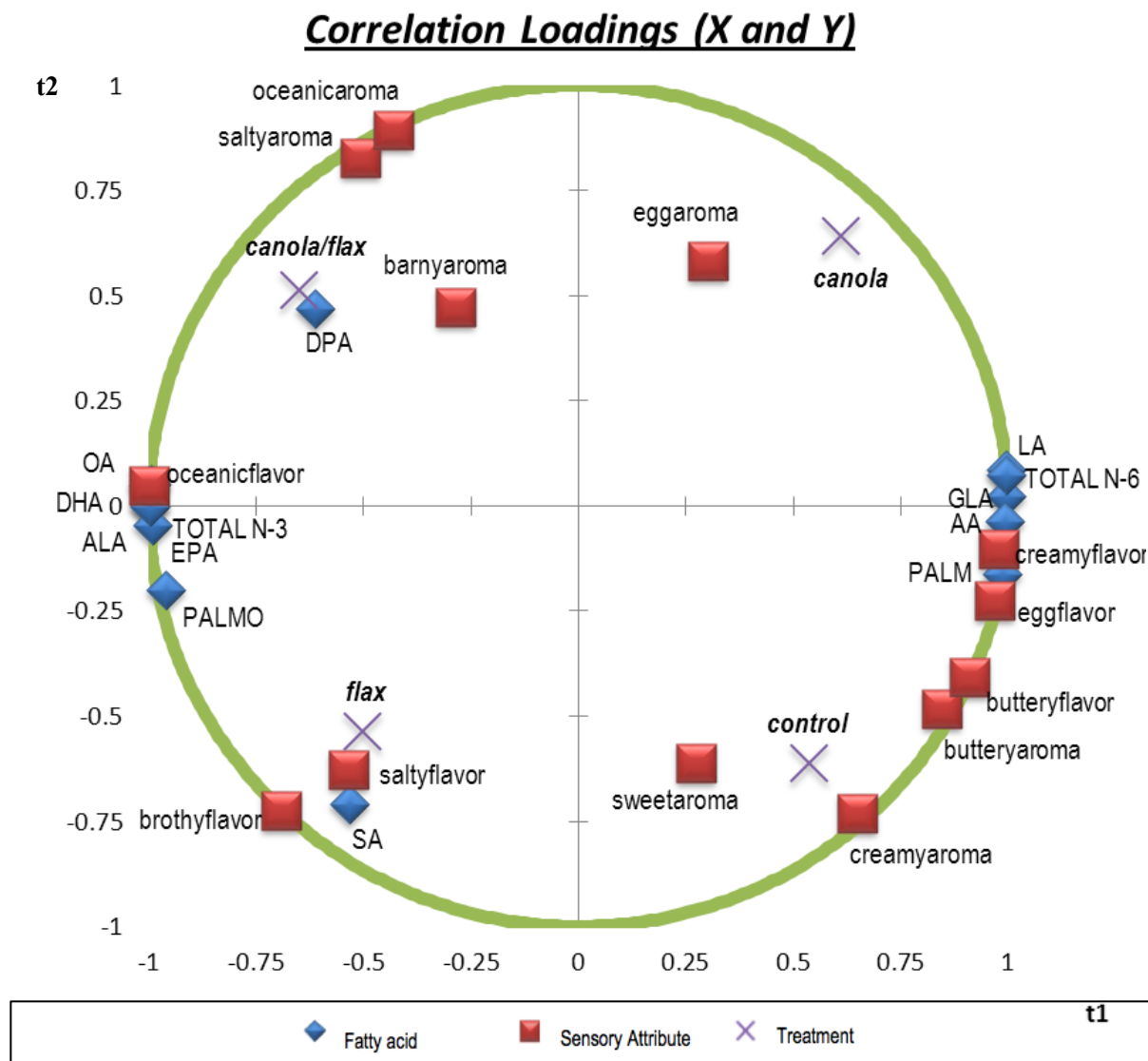
Figure 6.1: An overview of the correlation loadings from partial least squares analyses with yolk fatty acids as X-variables and sensory attributes of cooked egg samples as Y-variables from hens consuming canola meal, flaxseed oil or both

PALM= C16:0; PALMO= C16:1; SA= C18:0; OA= C18:1n9; LA= C18:2n6; GLA= C18:3n6; AA= C20:4n6; ALA= C18:3n3; EPA= C20:5n3; DPA= C22:5n3; DHA= C22:6n3; TOTAL n-6= C18:2n6 + C18:3n6 + C20:4n6; TOTAL n-3= C18:3n3 + C20:5n3 + C22:5n3 + C22:6n3.

6.5. Conclusions

To our knowledge, the present research is the first of its kind to investigate possible

interactions between CM and FO in laying hen diets on the fatty acid composition and sensory

quality of the resultant eggs. The present objective was addressed by testing egg yolks for fatty acid content and by utilizing a trained sensory panel to determine the aroma and flavour attribute intensities of cooked eggs. Overall, egg flavor was the only attribute to be significantly impacted through the interaction between CM and FO based on a lower mean score from descriptive analysis. Consumer testing is recommended to determine if this interaction results in decreased acceptability of eggs from chickens fed canola meal and flax oil.

Further investigation into the exact cause of taint warrants further research, especially since fishy taint is usually associated with fishy odor and not flavor and because aroma attributes were unaffected by dietary treatment in this study. In addition to fatty acids, volatile analysis should be done to understand the complexity of sensory outcomes in cooked eggs procured from designer diets. Eggs from other strains of laying hen should be tested in the same manner to determine the effect of genotype. Canola meal alone up to 24% of the diet will not alter the sensory profile, however careful consideration must be taken when combining it with n-3 ingredients, particularly FO as demonstrated in this study. Since the level of oil used in this study is quite high, it is unlikely that this effect will be achieved with a more typical inclusion level.

6.6. Acknowledgment

CHAPTER 7: GENERAL DISCUSSION

The main purpose of this dissertation was to gain a clearer understanding of the potential impact various layer diets have on resulting eggs in order to optimize egg fatty acid composition for human health, while balancing the effect on sensory quality. Laying hen capacity to convert ALA to LCPUFA is greater than in humans, the main purpose being to provide the chick with nutrients for proper development. There are a wide variety of dietary ingredients rich in n-3 PUFA that can be fed to laying hens to produce enriched eggs. There are also many factors that need to be carefully considered when formulating such diets in order to prevent sensory changes from occurring. It is essential that there be a balance between degree of enrichment, cost and feasibility if sensory quality is to be maintained compared to conventional eggs. Also, it is of the utmost importance to not sacrifice hen health, egg quality and production parameters while enriching these eggs.

The results from Chapter 3 revealed that HS and HO, two novel foods not yet approved by the CFIA in laying hen diets, are safe and effective ingredients. The levels used in these diets were at maximal inclusion levels to demonstrate that extraordinarily high levels of these ingredients do not pose any risk. Moreover, these two ingredients are rich in the beneficial n-3 PUFA and can subsequently enrich egg yolk with ALA, EPA and DHA, as well as increase yolk pigmentation suggestive of carotenoid enrichment. Based on fatty acid analysis, it was also observed that while ALA concentration increased linearly in the yolk, the LCPUFA DHA eventually reached a plateau, whereby further DHA enrichment was not possible (See Appendix B). With the knowledge that the enzymes D6DE and D5DE compete for ALA or LA for further desaturation into LCPUFAs, this led to the second study, which can be found in Chapter 4. In this study, we tested diets varying in LA content to determine whether or not decreasing LA in

the diets could alleviate the competition it had with ALA, leading to more DHA enrichment.

Unfortunately this was not the case, likely due to the levels of LA being too similar in the diets.

However, the subsequent study diets using coconut oil as a source of SFA to replace even more

LA resulted in greater ALA conversion. At the same time, n-6 PUFA were signficantly

decreased but SFA levels increased. Despite the negative history SFA has on indices of health, a

recent study found no association between dietary intake of SFA and incident coronary events or

mortality in patients with established coronary artery disease (Puaschitz and others 2015).

Perhaps the modest increase in SFA that we observed should not take away from the significant

increase in n-3, coupled with a reduction in n-6. It is our recommendation based on our results

that the egg industry focus on simultaneously increasing dietary n-3 PUFA and decreasing the

SFA: OA + LA ratio in order to maximize n-3 enrichment in the egg yolk.

Our final study aimed to assess the potential of CM to interact with a source of n-3

PUFA, in this case using FO, the most common enrichment source. It was our hypothesis that

CM could negatively interact with the n-3 PUFA in FO and result in a deleterious sensory profile

in cooked eggs. Combining these two ingredients resulted in decreased egg flavour compared to

using the ingredients in isolation. Based on the results, our suggestion for the egg industry is to

proceed with caution and carefully monitor the usage of choline-rich ingredients like CM when

designing functional feed for hens. Of course, a consumer panel would be needed to support this

conclusion. With the availability of molecular testing, and the inheritance pattern clear, breeding

companies have made great progress in removing tainting from their flocks over the last two

decades. The elimination of tainting hens from commercial lines have allowed egg producers to

feed large amounts of CM without worry of producing tainted eggs, and this has expanded export

markets for Canadian CM to egg producers around the world. However, results from our study

demonstrate that the use of CM in functional feeds to produce n-3 enriched eggs still remains a concern due to other factors that result in impaired sensory quality, irrespective of the defective FMAO gene.

There were a number of similarities between the PLS analyses from each of the trials. Firstly, there was always a distinct and clear separation between dietary treatments, indicative of differences between them in terms of sensory attributes and yolk FA. In 2 of the 3 sensory trials, egg flavour was more associated with n-3 PUFA. But there were many differences in PLS analyses between the different research trials, which can be attributed to different panelists and a different set of agreed upon attributes. For example, in the canola/flax trial, egg flavour was more associated with n-6 PUFA and less associated with n-3 PUFA. A possible explanation for this is because diets from this trial resulted in deleterious flavours whereas the other 2 trials did not, the observed oceanic flavour could have overpowered the egg flavour. In general, there are certain ingredient thresholds that cannot be surpassed if sensory quality is to be maintained, and this level was clearly reached at the inclusion of 7.5% FO in the layer diet.

As noted in the literature review, an 'n-3' egg can only be labeled as such if there is a minimum of 300 mg total n-3 PUFA in the egg (Health Canada 2012b). However, changing regulations outlining minimum nutrient content could impact the egg industry significantly. For example, new Canadian regulations will eventually require a serving size of 100 g, equivalent to approximately 2 eggs, to contain at least 300 mg total n-3 PUFA. This allows for greater flexibility in the design of hen diets. For example, according to current standards hemp-fed hens could not produce eggs with a nutrient content claim, but under the new guidelines eggs from hens fed at least 8% HO could bear a claim. This would have a very positive impact on hemp producers alike. Since there is always the potential for regulations to change, staying up-to-date

with current policies is critical.

Overall this research has demonstrated that there are definite limits in enrichment that can realistically be achieved through diet manipulations, particularly when using vegetarian ingredients. However, with careful diet manipulation it is possible to favorably shift the egg fatty acid composition.

CHAPTER 8: SUMMARY AND CONCLUSIONS

The overall objective of this thesis was to evaluate laying hen dietary interventions to increase n-3 PUFA content in eggs. The research assessed the effect of diet on egg fatty acid composition, sensory quality characteristics including appearance, aroma and flavour, and hen health and production. The following strengths and limitations of this research are outlined below:

1. Strengths

    a. The development of a novel cooking method for eggs should represent a new standardized method for egg preparation in sensory panels. This method is ideal for use in future studies to provide consistent results across time, and also allows for maximal retention of volatiles in samples that contribute to a heightened aroma perception.

    b. The collection of sensory data and egg yolk fatty acids allowed us to explore the relationships between dietary treatments, fatty acids which contribute greatly to sensory outcomes, and specific aroma and flavour attributes. This information can be used as a stepping stone for the development of future studies, as our results demonstrate that egg flavour is closely related to n-3 PUFA, while oceanic flavour may overpower this effect at a certain concentration of n-3 PUFA in the diet.

2. Limitations

    a. The high variability among panelists in the sensory test sessions is concerning as panelists should be working cohesively as an 'instrument' to assess the intensity

of a variety of sensory attributes. More training sessions are likely needed to decrease this variability further to provide reliable data.

b. Although the novel cooking method used in this research has a number of benefits, this technique is not commonly used among consumers. While the data we obtained from trained sensory panels was useful for our research objectives, the use of consumer panels are needed to correlate findings, and also to determine consumer acceptability.

The following conclusions could be drawn from the current research:

1. Maximal dietary inclusion of HO and HS:

a. Had mimimal effect on the sensory profile of cooked eggs, with the exception that eggs from the 4% HO group were significantly less sweet compared to eggs from the 20% HS group

b. Significantly increased total n-3 PUFA, and decreased n-6 PUFA in yolk

c. Resulted in a DHA plateau being reached in egg yolk FA

d. Significantly enhanced yolk colour

2. Dietary designer oil blends:

a. Resulted in an increase in total n-6 PUFA and DPA, and a decrease in OA and EPA in yolk with increased dietary LA

b. Resulted in an increase in LAUR, ALA, EPA, DPA, DHA, and a decrease in LA and AA with an increased dietary SFA: LA + OA ratio

c. Did not overcome the DHA plateau that was observed in the hemp trial when dietary ALA was increased from 15 to 30% (expressed as a percentage of total FA)

        d. Did not affect the sensory profile of cooked eggs

3. Dietary CM combined with FO:

        a. Resulted in minimal changes to yolk FA, with the exception of a significant

            interaction for LA and total n-6 PUFA

        b. Resulted in a significant decreased intensity of egg flavour

CHAPTER 9: FUTURE RESEARCH DIRECTIONS

There is increasing consumer demand for functional eggs and egg products. Because of this demand, prices are decreasing for specialty eggs and more consumers are opting to choose such eggs over conventional for a variety of reasons. Based on the results from this research, it is evident that there is potential for the egg industry to manipulate layer diets in order to enhance egg nutritional value while maintaining acceptability.

Because individual thresholds for certain attributes vary greatly, it is important to consider untrained (consumer) panels for the future. In selecting trained panels to fulfill our research objectives we have gained an understanding of potential attributes that could arise when designing new dietary interventions for laying hens. This was especially important since we were testing novel ingredients like hemp products and did not know what to expect. Additionally, trained panelists do not consider likeability in their assessment, as this would skew other variables we are testing or trying to measure accurately. However, it is unknown whether or not a general population would deem these eggs palatable and thus, acceptable. Of course, lack of acceptability would result in negative purchase behavior. Thus both types of panels are needed to truly understand the extent to which dietary treatments impact egg quality. Furthermore, we developed the egg cooking method in a manner that has not been done in previous research. We feel that this method offered clear advantages over standard cooking methods and was useful in meeting our research objectives. Since this method is not typical of what a consumer would use at home, a variety of commonly used cooking methods should be tested. Consumers typically do not have access to freshly laid eggs as those used in our studies,

therefore aged eggs should also be tested since volatile composition may change over time that can negatively effect sensory outcomes.

Novel ingredients used in this research including HS resulted in a significant increase in yolk pigmentation, which suggests subsequent carotenoid elevations. Because carotenoid absorption is increased in the human intestine when consumed in conjunction with lipids, eggs may act as an effective delivery system for some carotenoids (Olson, Ward, Koutsos 2008). Therefore, the concentration and profile of carotenoids in the yolks should be determined in order to confirm that desirable carotenoids are significantly increased.

The current approach for enriching eggs in industry is relatively crude and untargeted. Essentially a certain level of an n-3 rich ingredient like flax or fish oil is added to the diet to boost subsequent levels in the eggs. However, it would be quite naïve to think that only the fatty acid profile is being altered. Unfortunately, highly standardized methods of lipid analysis have yet to be established, but many different techniques based on chromatography, mass spectrometry and spectroscopy are currently available (Fuchs and others 2007). Mass spectrometry is regarded as one of the most powerful analytical methods for lipid analysis (Watson 2006), and the use of high-throughput technology such as HPLC, UHPLC and MALDI-TOF MS has been used to determine xanthophylls (Brulc and others 2013; Schlatterer and Breithaupt 2006), cholesterol (Albuquerque and others 2014) and phospholipids (Fuchs and others 2007) in egg yolk. Metabolomics is the systematic study of the unique chemical fingerprints that specific cellular processes leave behind (Daviss 2005). A non-targeted approach aims to cover the metabolome as broadly as possible, making it an unbiased method. In doing so, characterization of both known compounds and previously unknown or poorly characterized metabolites is possible (Oresic 2009). Using a metabolomics approach, we can

further analyze egg components to understand the impact of altering the hen diet. Furthermore, it is well known that hundreds of volatile compounds contribute to the overall sensory experience of foods. Some of these compounds have been identified in whole egg, yolk and albumen (Warren, Larick, Ball 1995). However, there are likely thousands of other compounds in egg that may impact sensory quality. The extent to which different diets affects the aroma and flavour profile of eggs is unknown.

An instrument like LC-QTOF-MS can be used to rapidly characterize the composition of biological samples such as blood and urine, however, there is great potential for its use in food products like eggs, also a biological product. We wanted to further explore this avenue of promising research because eggs have never been tested using this approach. In Appendix Q, preliminary results of method development used to characterize egg yolk compounds using a non-targeted metabolomics approach are outlined. The confirmation of compounds using MS/MS and the creation of a yolk compound database is important for the future of the egg industry to have more control over the egg composition, so researchers can more effectively design layer diets to deliver functional eggs to target populations. It is also imperative that more research is done to gain a better understanding of the physiological effects of different structural variations of lipids, rather than just free FA. With such information, a more targeted approach can be utilized to develop other functional eggs and egg products for niche markets. At the same time, the sensory characteristics may be linked to compounds in yolk that can help to further explain the sensory profiles we observed in cooked eggs from different dietary treatments. Further research is needed to construct an exhaustive yolk database, and thereafter the health benefits of designer eggs should be assessed using human clinical trials in order to determine biological effects of consuming these eggs.

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APPENDICES

A: Animal ethics approval

UNIVERSITY OF MANITOBA | Research Ethics and Compliance
Office of the Vice-President (Research and International)

Animal Care & Veterinary Se
208-194 Dafoe Road
Winnipeg, MB
Canada R3T 2N2
Phone +204-474-8880
Fax +204-269-7173
veterinaryservices@umanitob

23 November 2012

TO: Dr. J. House, Department of Human Nutritional Sciences

FROM: Dr. H. Aukema, Chair, Fort Garry Campus Animal Care Committee

RE: **Renewal of Protocol F11-044**

Please be advised that your request for renewal of your project entitled **"Assessment of the Safety and Efficacy of Hemp Seed Products for Use as Feed Ingredients in Poultry Diets"** (category of invasiveness "B"), has been approved and is valid from **January 1 2013 to December 31 2013. The reference number for this protocol is now F11-044/1.** The protocol reference number must be used when ordering animals.

Please be reminded that abbreviations should be spelled out the first time they are used in the document. (e.g. CFIA, AMEn)

It is understood that these animals will be used only as described in your protocol. The protocol must be kept current. Should changes become necessary, very minor alterations can be made with the approval of the University Veterinarian, provided that the protocol in Central files is amended appropriately. More substantive changes will require resubmission to and reassessment by the Fort Garry Campus Animal Care Committee. If approved, this will result in the assignment of a new protocol reference number.

Failure to follow this protocol, or renew it prior to the expiry date, will result in the termination of your ability to continue using or ordering animals. **Please be advised that only three renewals are allowed. Subsequently, a full application must be submitted.**

B: Laying hen intake of ALA (X-axis) and yolk n-3 fatty acid composition (Y-axis) of eggs from hens fed hempseed or hempseed oil

$y = 0.1385x - 6.1909$
$R^2 = 0.86148$

$y = 0.002x + 0.1604$
$R^2 = 0.5354$

$$y = 0.004x + 2.0047$$
$$R^2 = 0.35466$$

$$y = 13.296\ln(x) - 45.159$$
$$R^2 = 0.70382$$
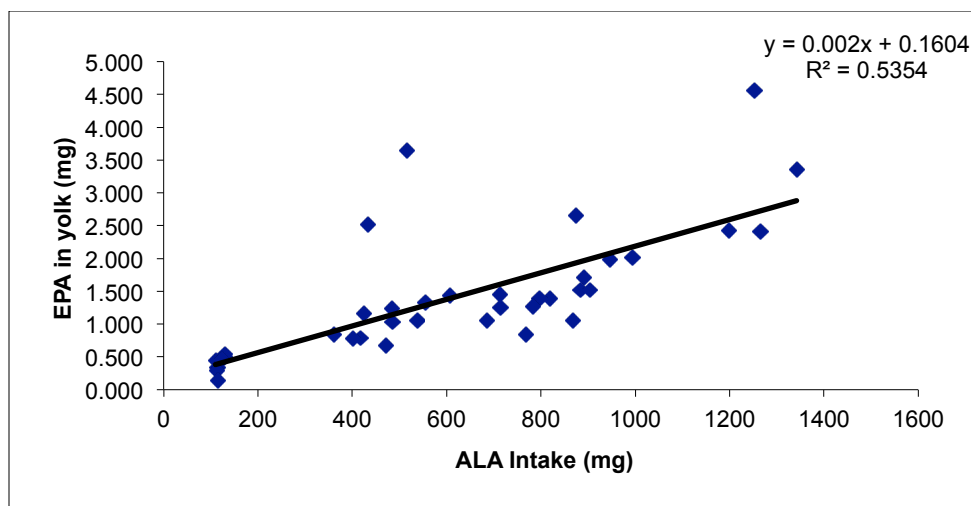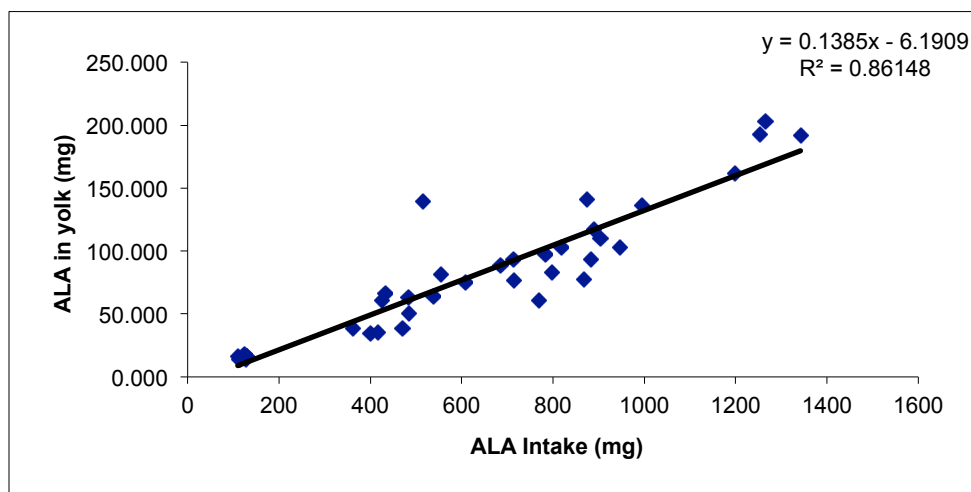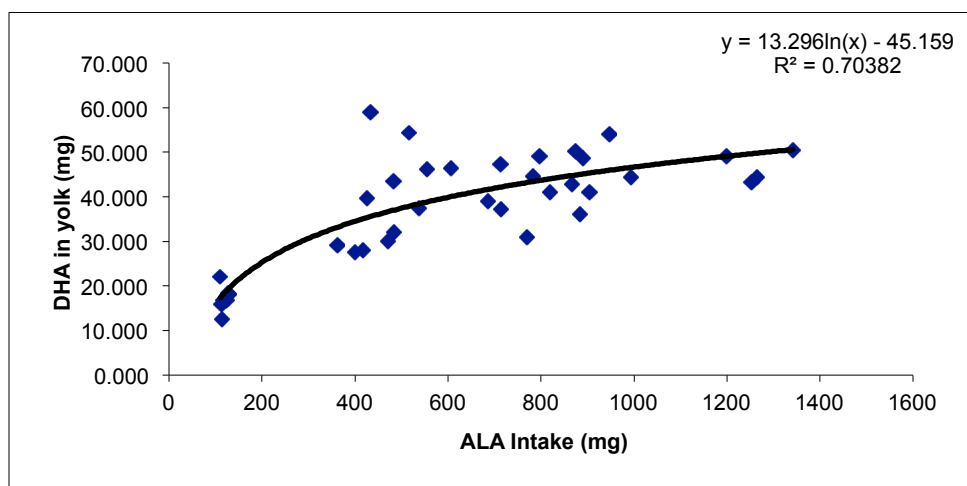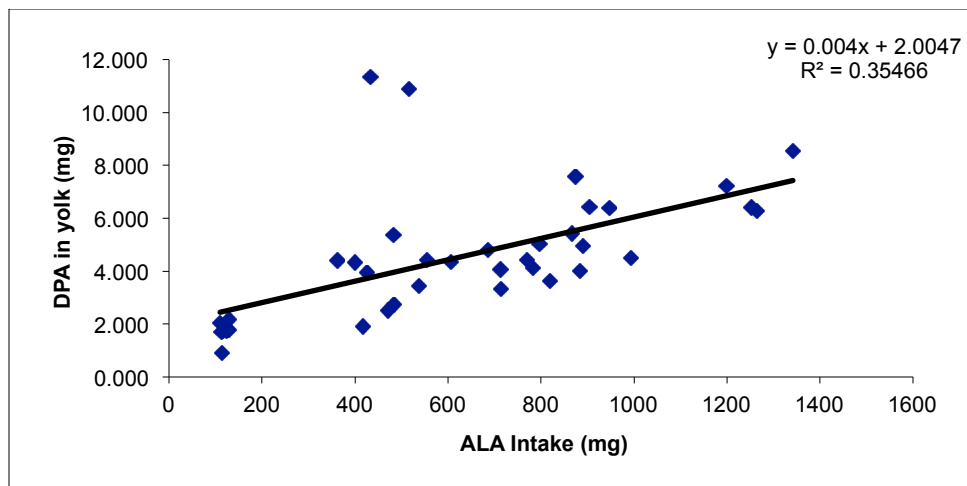
C: Human ethics approval certificate

UNIVERSITY | Ethics
OF MANITOBA | Office of the Vice-President (Research)

CTC Building
208 - 194 Dafoe Road
Winnipeg, MB R3T 2N2
Fax (204) 269-7173
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APPROVAL CERTIFICATE

June 8, 2010

J. House
Province of Manitoba, Science, Technology
Energy & Mines
Manitoba Science and Technology International
Collaboration Fund

TO: **Michael Aliani**
Principal Investigator

FROM: **Wayne Taylor, Chair**
Joint-Faculty Research Ethics Board (JFREB)

Re: Protocol #J2010:067
"Sensory Evaluation of Scrambled Eggs From Hens Fed Designer Oils"

Please be advised that your above-referenced protocol has received human ethics approval by the **Joint-Faculty Research Ethics Board**, which is organized and operates according to the Tri-Council Policy Statement. This approval is valid for one year only.

Any significant changes of the protocol and/or informed consent form should be reported to the Human Ethics Secretariat in advance of implementation of such changes.

Please note:

- if you have funds pending human ethics approval, the auditor requires that you submit a copy of this Approval Certificate to Eveline Saurette in the Office of Research Services, (e-mail eveline_saurette@umanitoba.ca, or fax 261-0325), including the Sponsor name, before your account can be opened.

- if you have received multi-year funding for this research, responsibility lies with you to apply for and obtain Renewal Approval at the expiry of the initial one-year approval; otherwise the account will be locked.

D: Human ethics renewal approval

UNIVERSITY | Ethics
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RENEWAL APPROVAL

May 27, 2011

TO: Michel Aliani
           Principal Investigator

FROM: Brian Barth, Chair
           Joint-Faculty Research Ethics Board (JFREB)

Re: Protocol #J2010:067
           "Sensory Evaluation of Scrambled Eggs from Hens fed
           Designer Oils"

Please be advised that your above-referenced protocol has received approval for renewal by the **Joint-Faculty Research Ethics Board**. This approval is for one year only.

Any significant changes of the protocol and/or informed consent form should be reported to the Human Ethics Secretariat in advance of implementation of such changes.

E: Human ethics amendment approval

UNIVERSITY OF MANITOBA | Research Ethics and Compliance
Office of the Vice-President (Research and International)

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AMENDMENT APPROVAL

August 13, 2012

TO: Michael Aliani
Principal Investigator

FROM: Wayne Taylor, Chair
Joint-Faculty Research Ethics Board (JFREB)

Re: Protocol #J2010 :067
"Sensory Evaluation of Scrambled Eggs from Hens Fed Designer Oils"

This will acknowledge your request dated August 1, 2012 requesting amendment to your above-noted protocol.

Approval is given for this amendment. Any further changes to the protocol must be reported to the Human Ethics Secretariat in advance of implementation.

F: Human ethics renewal approval

UNIVERSITY OF MANITOBA | Research Ethics and Compliance
Office of the Vice-President (Research and International)

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RENEWAL APPROVAL

MB Science, Technology, Energy and Mines, and Manitoba Science and Technology International Collaboration Fund

August 13, 2012

TO: **Michael Aliani**
Principal Investigator

FROM: **Wayne Taylor, Chair**
Joint-Faculty Research Ethics Board (JFREB)

Re: Protocol #J2010 :067
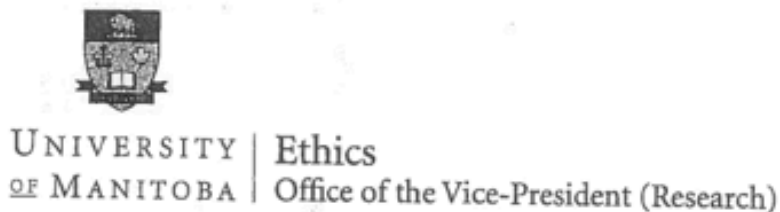"Sensory Evaluation of Scrambled Eggs from Hens Fed Designer Oils"

Please be advised that your above-referenced protocol has received approval for renewal by the **Joint-Faculty Research Ethics Board**. This approval is for one year only.

Any significant changes of the protocol and/or informed consent form should be reported to the Human Ethics Secretariat in advance of implementation of such changes.

G: Recruitment email for sensory panelists

Hello Everyone,

There is an opportunity to become involved in Food Sensory Research in the Faculty. Aroma and flavor attribute analysis of cooked eggs is the focus.
Details are outlined in the attached information package which includes a letter, consent form and questionnaire. The panel sessions will be held in the Weston Sensory Centre Fourth Floor Human Ecology Building with an honorarium provided.

A response by XXX would be greatly appreciated as we are tentatively planning the first session for XXXX from 11:30 to 12:15.

Forwarding this e-mail to colleagues that may be interested would be greatly appreciated.

Department Office Assistants: Could you please forward this e-mail to graduate students and sessional instructors?

Thank you very much for considering our request.

Sincerely,

███████████, Department of Human Nutritional Sciences
Weston Sensory and Food Research Centre
████████████████████████

and

███████████, Department of Human Nutritional Sciences
Weston Sensory and Food Research Centre
████████████████████████

H: Recruitment letter for sensory panelists

On Department of Human Nutritional Sciences Letterhead

Date

Dear Colleague,

We are recruiting volunteers to participate in a research study on the aroma and flavor of cooked eggs. You would have the opportunity to learn how panelists are trained to measure the intensity of specific aroma and flavor attributes of this product. This letter explains what your commitment would be. If you have any questions please call me, ████████████████████ ████████████████████.

Eight to ten panelists will take part in group sessions (training component) followed by sessions where evaluation is done individually. Training involves group discussion of definitions and techniques for measuring flavor and textural attributes and evaluation of their intensities in various samples. Other food products may be used to facilitate these discussions and aid in the development of the measuring instrument. There will be six to eight training sessions and six individual sessions held from 11:30 to 12:15 during a five to six week period. The first meeting is tentatively planned for XXXXX when times and dates for future sessions will be confirmed with the group. A gift card from the University of Manitoba Bookstore for $70.00 will be given to those completing all of the required sessions. The study will take place on the Fourth Floor in the Human Ecology Building.

A potential risk would be allergic reactions to food products. Completion of the enclosed questionnaire stating known allergies will alert the researcher to possible risk. Respondents with allergies will be notified that they will not be allowed to participate in the study. If you are interested in helping us with this research notify ████ at ████████████ ████████████████ before XXXX. Please complete the attached consent form and questionnaire and e-mail them back before the first meeting.

We hope that you will be able to take part in this research and look forward to hearing from you. Alternatively, if you know of anyone else that might be interested in participating we would appreciate it if you could forward this information to them. Thank you.

Sincerely,

████████████, MSc
Sensory Evaluation Specialist for,

████████████, Assistant Professor
Department of Human Nutritional Sciences

I: Recruitment poster for sensory panelists

INTERESTED IN FOOD QUALITY?
VOLUNTEERS ARE NEEDED FOR
COOKED EGG TASTING

Where: Fourth Floor Human Ecology Building
Start Date and Time: X X X X - 11:30 to 12:15
Commitment: Group and Individual Sessions
      - approx. 3 per week for 5 to 6
       weeks

Honorarium Provided
Contact: ph   or e-mail
          for more information.

J: Consent form for sensory panelists

On Department of Human Nutritional Sciences Letterhead

Research Project Title: **Sensory Evaluation of Cooked Eggs From Hens Fed** ███████
Sponsored by: ███████████████████████████
███████████████████████████

Researchers: ████████████████████, Department of Human Nutritional Sciences

This consent form, a copy of which will be left with you for your records and reference, is only part of the process of informed consent. It should give you the basic idea of what the research is about and what your participation will involve. If you would like more detail about something mentioned here, or information not included, you should feel free to ask. Please take the time to read this carefully and to understand any accompanying information.

The study is being done to evaluate the aroma and flavor attributes of cooked eggs. Six to eight training sessions will be conducted where panelists meet as a group to learn the aroma and flavor attributes as well as the scale used to measure the intensity of the attributes. Samples of cooked eggs as well as other products useful in defining specific attributes will be tasted to familiarize panelists with procedures. Approximately six test sessions will be held in individual booths. All sessions will be approximately 45 minutes and take place on the Fourth Floor of the Human Ecology Building. The total time commitment will be up to 10.5 hours over a 5 to 6 week period.

Possible risk may be allergic reactions to food products eaten. Completion of the accompanying questionnaire will alert the researchers to any potential risk.

Panelists will be identified by number and all data related to personal information and results obtained will be kept in a locked cabinet in Room 400 Human Ecology Building for 5 years or until data are published whichever comes first. Access to information linking panelist to number will be limited strictly to the researchers named above. All data will be shredded after the time has expired. Data published will be given as group means with no individual names given.

If requested at the end of this form, you will receive a copy of the purpose of the study as well as the results.

A gift card from the University of Manitoba Bookstore for $70.00 will be provided for those completing all of the required sessions.

Your signature on this form indicates that you have understood to your satisfaction the information regarding participation in the research project and agree to participate as a subject. In no way does this waive your legal rights nor release researchers, sponsors, or involved institutions from their legal and professional responsibilities. You are free to withdraw from the study at any time, and/or refrain from answering any questions you prefer to omit, without prejudice or consequence. Your continued participation should be as informed as your initial consent, so you should feel free to ask for clarification or new information throughout your participation. This study is being conducted by ████████, Assistant Professor, Department of Human Nutritional Sciences, telephone – ███████, e-mail – ████████████████.

This research has been approved by the Joint-Faculty Research Board of Ethical Review at the University of Manitoba.   If you have any concerns or complaints about this project, you may contact the above-named person or the Human Ethics Secretariat at 474-7122.

_____

　　　　　Participant's Signature　　　　　　　　　　　　Date

Telephone Number _____　E-mail Address _____

_____

Researcher and/or Delegate's Signature　　　　　Date

Panelist Number _____

I would like a copy of the purpose and the results of the study sent to the e-mail address noted above   Yes _____   No _____

Delegate's contact information:

██████████, Sensory Evaluation Specialist
Room 400 Human Ecology Building
████████████████

K: Questionnaire for sensory panelists

This information will be kept strictly confidential.

Panelist # _____

1. Have you participated on sensory evaluation panels before?
Yes_____ No _____ If yes,

 a) What product(s) did you evaluate?

 _____

 _____

 b) Was training part of the evaluation procedure? Yes _____ No _____
If yes, indicate for which product(s).

 _____

 _____

2. Are you allergic to any food products? Yes _____ No _____
 If yes, note them below.

 _____

 _____

3. Are there any foods specifically, or food flavors and textures generally, that you would prefer
not to evaluate?

 _____

 _____

Thank you very much for completing this questionnaire.

L: Sensory training orientation information

Sensory Evaluation – Egg Study
Orientation

Purpose
Generally, our task is to measure the intensity of the sensory attributes of eggs.

PART 1 - Training
Activities
1.   Learn the methods/techniques to evaluate the aroma and flavor attributes.

2.   Demonstrate with standard samples the attribute definitions.

3.   Measure the intensity of the attributes of a number of egg samples on a line scale.

Outcome
Individually, panelists are evaluating the samples consistently over time.
Within the group, panelists are evaluating the samples consistently over time.

Goal
Reduce variability for data collected.

PART 2 – Experimental Samples

Responsibilities
Panelist 1.   Come to panels on time so that we are not held up and can leave on time. 2.   Notify experimenter if unable to attend, and make up the session at a convenient time. 3.   Refrain from using perfume, strong soaps/shampoos, cosmetics, etc. on panel days so that senses are maximized. 4.   Refrain from eating, drinking anything but water and smoking 30 minutes prior to the panel. **Leader** 1.   Have panel ready on time. 2.   Organize work to complete during the time allotted. 3.   Notify panelists regarding the schedule well in advance. 4.   Make sure everyone is comfortable.

M: Panelist instructions for evaluating aroma and flavour

Instructions for Egg Panelists

AROMA EVALUATION
Place the sample container in position for sniffing.
Remove the cover.
Take three short sniffs and replace the cover.

FLAVOR EVALUATION
Take about one half teaspoon of sample.
Chew the sample thoroughly.

N: Examples of ballots used in training sessions

Egg Evaluation   Panelist # _____

Please note descriptors for the aroma and flavor of the samples in the space provided below. Additional comments are welcome.

Code Number	Aroma	Flavor	Comments

Panelist # _____

Egg Evaluation

Place a vertical line across the horizontal line at the point that best describes the intensity of the attribute present in the sample. Note the sample code number above the vertical line.
Evaluate the samples in the following order:

_____   _____   _____   _____   _____   _____

AROMA
Remove the lid from the sample container.
Take three short sniffs and replace the lid.

Sweet

_____
low   high

Egg

_____
low   high

Sour

_____
low   high

FLAVOR
Taste the egg sample and evaluate the following taste/flavor attributes.
Sweet

_____
low   high

Egg

_____
low   high

Sour

_____
low   high

O: Panelist instructions for evaluating aroma and flavour in test sessions

Egg Panelist Instructions

Procedures for Evaluating Aroma and Flavor

AROMA
Place the sample container in position for sniffing.
Remove the cover.
Take three short sniffs and replace the cover.

FLAVOR
Take about ½ teaspoon of sample and place in your mouth.
Chew the sample thoroughly.
Evaluate the flavor attribute intensities just before swallowing the sample.

Evaluate the AROMA ATTRIBUTES FIRST for all of the samples in the order they are presented across the top of the grid. When all of the samples are evaluated for aroma go back to the first sample and evaluate the flavor in the same order.

Left double click on the box corresponding to the sample code and the attribute to evaluate.
The line scale will appear
Click on the line at the point which corresponds to the intensity of the attribute.
Click on the box to remove the line. Proceed to the box corresponding to the next attribute.
To make a comment click on the notebook icon in the box and use the keyboard or the screen to enter your response. Click on accept/return to exit the comment option.
When all of the boxes in the grid are completed click on the END sign.

P: Example of sensory statistical analysis, if a significant panelist-by-treatment interaction was observed, the main effects of panelist and treatment were tested by the interaction effect

	df	SS	Mean SS	Versus error		Versus interaction	
				F	prob	F	prob
Treatment	5	41.11	8.22	8.22/3.60 =2.28	NS	8.22/6.09 =1.35	NS
Panelist	7	509.57	72.80	72.80/3.60 =20.22	0.000	72.80/6.09 =11.95	0.000
Interaction	35	213.10	6.09	1.69	0.031		

Q: METABOLOMIC PROFILING OF EGG YOLK USING LIQUID CHROMATOGRAPHY QUADRUPOLE TIME-OF-FLIGHT MASS SPECTROMETRY

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Abstract

A simple and sensitive method for the separation of compounds found in egg yolk was developed using LC-QTOF-MS. White Lohmann hens of 60 weeks of age were fed one of four dietary treatments, containing 24% CM, 7.5% FO, both or neither (control). Extracts were prepared from frozen egg yolks procured from these hens, and analyzed using an HPLC system coupled to a Q-TOF LC/MS equipped with a dual electrospray ionization source. Over 3872 metabolites were detected in egg yolk extracts, of which 764 entities were found after filtering with a cut-off percentage of 50%. One-way ANOVA revealed a total of 26 entities that were significantly different ($P< 0.05$) among the 4 treatments, of which 14 could be identified through a database. Further studies using mass MS/MS and nuclear magnetic resonance (NMR) would need to be conducted in order to validate these compounds. Additionally, the development of a database for egg yolk compounds will be useful for the future in designing tailored diets to target specific compounds.

Introduction

Hen eggs are an important source of essential nutrients, consisting of approximately 74% water, 12% protein and 11% lipids (Belitz, Grosch, Schieberle 2009). The unique composition of the egg yolk consists of approximately 31% lipids mostly in the form of TG (66%) and PL (28%), which are organized in the form of low-density lipoproteins (68%), high-density lipoproteins (16%), livetins (10%) and phosvitins (4%) (Belitz, Grosch, Schieberle 2009). About 5% of the egg lipids consist of sterols, including cholesterol (96%), of which 15% is esterified with FA. Other components of the sterol fraction include cholestanol, 7-cholestenol, campesterol, β-sitosterol, 24-methylene cholesterol and lanosterol.

The basic composition of eggs described above deviates to some degree, possibly as a result of hen age and breed, however certain compounds in egg yolk are highly responsive to manipulations in the hen diet (Bertechini and Mazzuco 2013). The use of designer hen diets to enrich eggs in various bioactive compounds has been successful and widely used in industry, including the incorporation of desirable lipids (n-3 PUFA) (Cherian and others 2013; Fraeye and others 2012; Goldberg and others 2012), pigments (lutein) (Leeson and Caston 2004), vitamins (folate, vitamins A, E, D) (Grobas and others 2001; Schiavone and others 2011; Yao and others 2013) and minerals (iron, selenium, zinc) (Schiavone and others 2011; Surai and Sparks 2001). Subsequently, desirable lipids such as n-3 PUFA can be incorporated into egg yolk at higher levels by including ingredients such as flax, hemp, canola, fish and algal products into hen feed. Depending on the dietary feed ingredient used, varying levels of n-3 PUFA can be achieved, as reviewed by Fraeye and others (2012), while SFA and MUFA in eggs are hardly influenced (Baucells and others 2000; Cachaldora and others 2008a; Herber and Van Elswyk 1996; Van Elswyk, Dawson, Sams 1995). Naturally occurring PL, from plant or animal origin,

predominantly contain an unsaturated FA in the sn-2 position and a saturated FA in the sn-1 position (Cohn and others 2010). Furthermore, it has been observed that the fatty acid pattern of the feed is reflected more clearly in the TG fraction (neutral lipids) of egg lipids than in the PL fraction (polar lipids) (Belitz, Grosch, Schieberle 2009). At the same time, LCPUFA such as DHA are selectively incorporated into yolk PL, compared to TG, irrespective of the form of the lipids being consumed (Fredriksson, Elwinger, Pickova 2006; Schreiner and others 2004). The PUFA content of the sn-2 position of the PL cannot exceed a certain amount; thereafter the n-3 LCPUFA will be increasingly stored in the TG fraction (Schreiner and others 2004). Further research has demonstrated that although n-3 PUFA that are predominantly linked in the sn-1 and sn-3 position of TG from the diet, they were esterified in the sn-2 position of the TG and PL of egg yolk obtained after feeding laying hens with enriched diets (Pacetti and others 2005).

Unfortunately current methods of determining yolk composition are a) time consuming and expensive, since not just one method needs to be employed and b) highly variable since the use of multiple methodologies between laboratories provide inconsistent results. Although numerous methods for assessing the positional distribution of FA in each lipid class are available, there has not yet been a method described in the literature to detect all of these yolk components in a single method. The extent to which variations in dietary treatments affect the positional distributions of FA in each TG and PL is unknown.

Therefore, developing and optimizing a sensitive, quick method to characterize the array of compounds in egg yolk can be very useful for industry in order to create new functional eggs, maintain consistency and investigate potential quality problems that may arise. The objective of this research is to develop a non-targeted LC-QTOF-MS method capable of monitoring metabolites in egg yolk from eggs procured from hens fed four different diets. This research will

also provide the information needed to design appropriate dietary strategies to deliver targeted compounds using functional eggs as a vehicle.

Materials and Methods

Diets

White Lohmann hens of 60 weeks of age were fed one of four dietary treatments. Diets were arranged in a 2 x 2 factorial design, containing 24% CM, 7.5% FO, both or neither (control). Ingredient composition of the diets can be found in Table Q1. Eggs were procured from these hens after 4 weeks of feeding.

Table Q1: Ingredient composition of layer diets containing canola meal, flaxseed oil or both

Ingredients (%)	Control	Canola Meal	Flax Oil	Canola & Flax
Ground canola meal	0.00	23.63	0.00	23.63
Ground soybean meal	17.60	0.00	17.60	0.00
Wheat	59.85	58.11	59.85	58.11
VM Premix[1]	2.50	2.50	2.50	2.50
Salt	0.30	0.30	0.30	0.30
Limestone	10.19	10.02	10.19	10.02
Dicalcium phosphate	1.68	1.40	1.68	1.40
Lysine	0.21	0.32	0.21	0.32
DL-Methionine	0.16	0.10	0.16	0.10
Threonine	0.01	0.00	0.01	0.00
Corn oil	7.50	7.50	0.00	0.00
Flaxseed oil	0.00	0.00	7.50	7.50
Calculated Composition				
AMEn (Poultry; Kcal/kg)	3095.00	3121.06	3095.00	3121.06
Crude Fat (%)	9.24	9.66	9.24	9.66
Crude Protein (%)	18.50	18.50	18.50	18.50
Total Lysine (%)	0.98	1.00	0.98	1.00
Calcium (%)	4.10	4.10	4.10	4.10
Total Phosphorus (%)	0.67	0.77	0.67	0.77
Available Phosphorus (%)	0.45	0.45	0.45	0.45
Sodium (%)	0.15	0.15	0.15	0.15

Chloride (%)	0.22	0.22	0.22	0.22
Methionine (%)	0.40	0.40	0.40	0.40
Threonine (%)	0.60	0.60	0.60	0.60

[1]Provided per kg of diet: 11,000 IU vitamin A, 3000 IU vitamin D3, 150 IU vitamin E, 3 mg of vitamin K(as menadione), 0.02 mg cyanocobalamin, 6.5 mg riboflavin, 4 mg folic acid, 10 mg of calcium pantothenate, 40.1 mg niacin, 0.2 mg biotin, 2.2 mg thiamine, 4.5 mg pyridoxine, 1000 mg choline, 125 mg of ethoxyquin (antioxidant), 66 mg Mn (as manganese dioxide), 70 mg Zn (as zinc oxide), 80 mg Fe (ferrous sulphate), 10 mg Cu (as copper sulphate), 0.3 mg Se (as sodium selenite), 0.4 mg I (as calcium iodate), and 0.67 mg iodized salt. VM= Vitamin-Mineral; AMEn= Nitrogen corrected apparent metabolisable energy

Sample preparation

The following extraction procedure, which focuses on extracting predominantly lipophilic substances, was an adaptation from International Sorbent Technology. Fresh egg yolks were separated from albumin and placed into plastic bags, kneaded and kept at -20°C until extraction. Egg yolk (1g) was weighed into centrifuge tubes. Acetonitrile (10ml), hexane (20ml.) and sodium sulphate (1g) was added to the tubes, vortexed for 2 min and then centrifuged at 4000 rpm for 15 min at 4°C. The supernatant (16ml) was transferred into clean centrifuge tubes, dried under nitrogen then reconstituted into hexane (4ml). One milliliter of this solution was transferred to another tube. Two hundred µl was transferred into another tube, and 800µl hexane was added and vortexed. After, 500µl was transferred into a clean tube, dried, then reconstituted in 1ml of 50:50:0.1 acetonitrile: water: formic acid.

HPLC-QTOF-MS conditions

Metabolomics analysis was performed on a 1290 Infinity Agilent HPLC system coupled to a 6538 UHD Accurate Q-TOF LC/MS from Agilent Technologies (CA, USA) equipped with a dual electrospray ionization (ESI) source. A 3x50 mm, 2.7µ Agilent Poroshell column (Agilent Technologies) was used to separate metabolites while the column temperature was maintained was at 60°C. The mobile phases A and B were water and acetonitrile, with 0.1% formic acid. A

sample size of 2 μL was injected by maintaining the HPLC flow rate at 0.7 mL/min with a

gradient program of: 0, 0.5, 16, 17 and 22 min with 30, 30 100, 100 and 30% of solvent B,

respectively. A post-run time of 2 min was buffered before injecting the next sample. The auto-

sampler was maintained at a temperature of 15°C. The mass detection was operated using dual

electrospray with reference ions of m/z 121.050873 and 922.009798 for positive mode; and m/z

119.03632 and 980.016375 for negative mode. The main parameters for MS were as followed:

gas temperature, 300°C; drying N_2 gas flow rate, 11L/min; Nebulizer pressure, 50 psig;

fragmentor voltage, 175V; skimmer voltage 50V and OCTRF Vpp voltage, 750V. Targeted

MS/MS mode was used to identify the potential biomarkers. As part of the MassHunter

Software, the collision energy was applied by setting an appropriate equation having a slope

value of 5 and offset value of 2.5. A full range mass scan from 50-3000 m/z with an extended

dynamic range of 2 GHz standardized at 3200 m/z was applied. Data acquisition rate was

maintained at the rate of 3 spectra/s at a time frame of 333.3 ms/spectra with a transient/spectrum

ratio of 1932. A summary of experimental details can be found in Table Q2.

Table Q2: Liquid chromatography quadruple time-of-flight mass spectrometry experimental details for analyzing yolk extracts from hens consuming canola meal, flaxseed oil or both

Parameters	*Details*	
Agilent 1290 Infinity LC System	Zorbax High Resolution HT SB-Aq column	
	(4.6 mm x 100 mm, 1.8 micron)	
Column Temperature	65ºC	
Mobile Phase	A: Deionized Water + 0.1% Formic Acid	
	B: Acetonitrile + 0.1% Formic Acid	
Gradient Conditions	Time (min)	% Mobile Phase B
	0	2
	8	98

	10	98
	12.5	2
Flow Rate	0.7 mL/min.	
Injection Volume	2 µL	
Needle Wash	100% Acetonitrile	
Agilent 6538 QTOF MS	Yolk Extracts	
Dry Gas Temperature	300°C	
Drying Gas Flow	11 L/min.	
Capillary Voltage	4000V	
Nebulizer Pressure	50 psig	
Fragmentor	175V	
Mass Range	m/z 50 to 1700	
Mode (ESI)	Positive	
QTOF Reference Solution	m/z 121.050873 m/z 922.00978	

Data Analysis

Metlin database for metabolites was used for data mining of untargeted LC-MS data using the Molecular Feature Extraction (MFE) in Agilent MassHunter Qualitative Analysis software (version B.05.00). Statistical analysis between dietary treatment extracts was performed using Agilent Mass Profiler Professional software (version 12.6). A one-way analysis of variance (ANOVA) for metabolites ($P<0.05$), fold change analysis (>2) and PLS were also performed.

Results and Discussion

This research is the first of its kind to use a QTOF system to characterize egg yolk compounds. Over 3872 metabolites were detected in egg yolk extracts, of which 764 entities were found after filtering with a cut-off percentage of 50%. One-way ANOVA unequal variance

(Welch) revealed a total of 26 entities (Table Q3) that were significantly different (P< 0.05)

among the 4 treatments. Of these 26, 14 could be identified through the database.

Table Q3: Identified compounds with a 2-fold difference among 50 significantly different (P<0.05) among eggs from hens consuming canola meal, flaxseed oil or both

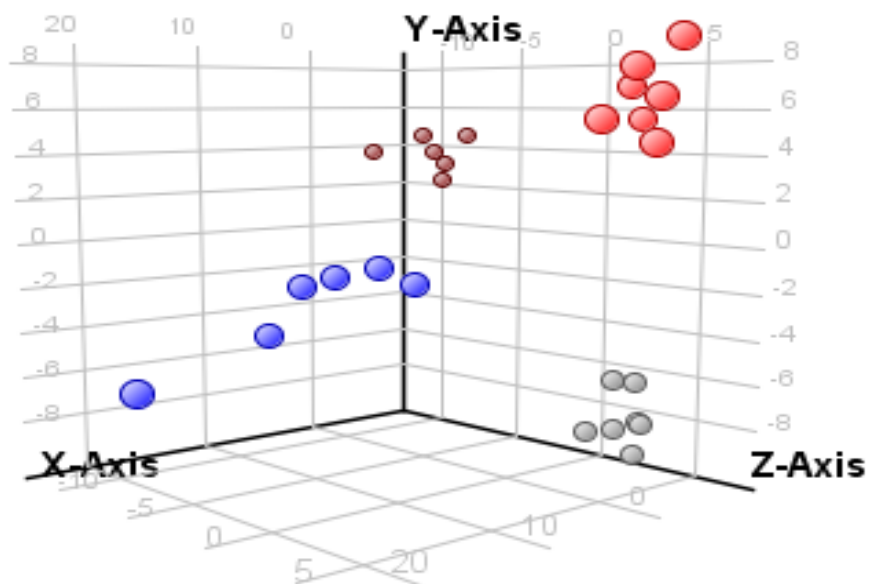| Metabolite[*]	Average raw values ± SD			
	Control	Canola	Flax	CanolaFlax
C16 H33 N	52×10^3 $\pm 2 \times 10^3$	55×10^3 $\pm 3 \times 10^3$	50×10^3 $\pm 1 \times 10^3$	50×10^3 $\pm 4 \times 10^3$
Lucidine B	30×10^4 $\pm 3 \times 10^4$	30×10^4 $\pm 1 \times 10^4$	28×10^4 $\pm 9 \times 10^3$	30×10^4 $\pm 6 \times 10^3$
C8 H4 O3	39×10^5 $\pm 3 \times 10^5$	39×10^5 $\pm 7 \times 10^4$	37×10^5 $\pm 1 \times 10^5$	38×10^5 $\pm 5 \times 10^4$
3-hexanoyl-NBD Cholesterol	84×10^5 $\pm 1 \times 10^6$	86×10^5 $\pm 4 \times 10^5$	87×10^5 $\pm 6 \times 10^5$	96×10^5 $\pm 3 \times 10^5$
PS(O-20:0/13:0)	11×10^5 $\pm 1 \times 10^5$	12×10^5 $\pm 5 \times 10^4$	11×10^5 $\pm 4 \times 10^4$	12×10^5 $\pm 3 \times 10^4$
4-Hydroxyphenylglyoxylate	42×10^4 $\pm 2 \times 10^4$	43×10^4 $\pm 2 \times 10^4$	40×10^4 $\pm 1 \times 10^4$	39×10^4 $\pm 1 \times 10^4$
C5 H10	12×10^4 $\pm 1 \times 10^4$	11×10^4 $\pm 9 \times 10^3$	11×10^4 $\pm 8 \times 10^3$	10×10^4 $\pm 8 \times 10^3$
C21 H37 N	21×10^4 $\pm 8 \times 10^3$	22×10^4 $\pm 6 \times 10^3$	20×10^4 $\pm 6 \times 10^3$	21×10^4 $\pm 4 \times 10^3$
C31 H55 N O15	34×10^4 $\pm 4 \times 10^4$	36×10^4 $\pm 2 \times 10^4$	36×10^4 $\pm 1 \times 10^4$	38×10^4 $\pm 2 \times 10^4$
C40 H26 Cl N9 O	34×10^3 $\pm 2 \times 10^4$	26×10^3 $\pm 3 \times 10^4$	66×10^3 $\pm 3 \times 10^3$	27×10^3 $\pm 3 \times 10^4$
C24 H47 N O	15×10^4 $\pm 2 \times 10^4$	13×10^4 $\pm 7 \times 10^3$	14×10^4 $\pm 6 \times 10^3$	14×10^4 $\pm 1 \times 10^4$
C7 H4 O2	12×10^4 $\pm 1 \times 10^4$	12×10^4 $\pm 9 \times 10^3$	11×10^4 $\pm 6 \times 10^3$	11×10^4 $\pm 3 \times 10^3$
Ascidiacyclamide	16×10^4 $\pm 9 \times 10^3$	16×10^4 $\pm 8 \times 10^3$	16×10^4 $\pm 5 \times 10^3$	18×10^4 $\pm 6 \times 10^3$
1289.6237@10.864264	39×10^4 $\pm 1 \times 10^4$	39×10^4 $\pm 1 \times 10^4$	40×10^4 $\pm 5 \times 10^3$	40×10^4 $\pm 1 \times 10^4$
Undecaprenyl phosphate α-L-Ara4FN	52×10^3 $\pm 3 \times 10^3$	48×10^3 $\pm 2 \times 10^3$	50×10^3 $\pm 3 \times 10^3$	50×10^3 $\pm 4 \times 10^3$
PE(19:0/20:2(11Z,14Z))	21×10^4 $\pm 4 \times 10^4$	18×10^4 $\pm 8 \times 10^4$	18×10^4 $\pm 1 \times 10^4$	19×10^4 $\pm 1 \times 10^4$
947.7125@13.493188	10×10^4 $\pm 9 \times 10^3$	11×10^4 $\pm 6 \times 10^3$	10×10^4 $\pm 3 \times 10^3$	10×10^4 $\pm 3 \times 10^3$
PS(O-20:0/17:0)	20×10^4 $\pm 2 \times 10^4$	20×10^4 $\pm 1 \times 10^4$	20×10^4 $\pm 1 \times 10^4$	22×10^4 $\pm 8 \times 10^3$

	21 x 10^5	22 x 10^5	22 x 10^5	23 x 10^5
PS(P-16:0/19:0)	± 2 x 10^5	± 7 x 10^4	± 7 x 10^4	± 9 x 10^4
	29 x 10^4	29 x 10^4	27 x 10^4	27 x 10^4
Lipid A -disaccharide-1-P	± 2 x 10^4	± 5 x 10^4	± 1 x 10^4	± 1 x 10^4
	87 x 10^3	79 x 10^3	80 x 10^3	78 x 10^3
Anthemis glycoside B	± 1 x 10^4	± 2 x 10^3	± 1 x 10^4	± 3 x 10^3
Formylmethionyl-leucyl-	14 x 10^4	15 x 10^4	12 x 10^4	11 x 10^4
phenylalanine methyl ester	± 6 x 10^4	± 1 x 10^4	± 9 x 10^3	± 5 x 10^4
	65 x 10^3	63 x 10^3	56 x 10^3	59 x 10^3
1060.388@17.898481	± 16 x 10^3	± 2 x 10^3	± 4 x 10^3	± 4 x 10^3
	13 x 10^4	14 x 10^4	13 x 10^4	15 x 10^4
770.2736@17.927822	± 5 x 10^4	± 9 x 10^3	± 7 x 10^3	± 7 x 10^3
PA(P-20:0/17:2(9Z,12Z))	20 x 10^4	28 x 10^4	20 x 10^4	32 x 10^4
ESI+175.0V 16.664515	± 1 x 10^5	± 1 x 10^5	± 1 x 10^5	± 3 x 10^4
PA(P-	43 x 10^3	51 x 10^3	24 x 10^3	14 x 10^3
20:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	± 2 x 10^4	± 3 x 10^4	± 3 x 10^4	± 2 x 10^4

[*]Metabolites identified by matching their Mass Spectra collected in electrospray ionization in positive mode (ESI+) against existing databases (Metlin and SimLipids)

Based on Figure Q1, it can be observed that there is a clear separation between the 4 treatments, suggesting differences among them in terms of their metabolites.

Legend - t-Scores

Color by All 4 groups Feeding Oil (Non-averaged)

🟥 [CanolaMeal]
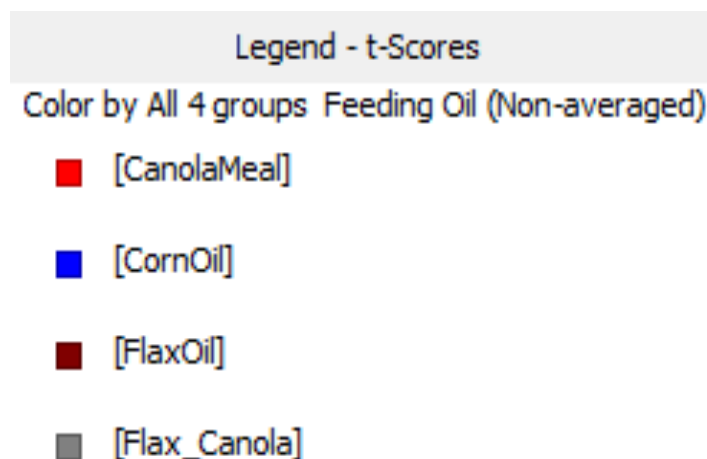
🟦 [CornOil]

🟥 [FlaxOil]

⬜ [Flax_Canola]

Figure Q1: An overview of the loading from partial least squares analyses with egg yolk metabolites to investigate the effect of dietary treatments containing canola meal, flaxseed oil or both

Note: Each circle on the graph represents one egg randomly chosen from the pool of eggs within the corresponding treatment.

Canola meal naturally contains anti-nutritive factors including glucosinolates (goitrogenic), erucic acid (toxic), tannins, sinapine, phytic acid and mucilage. It is possible that these compounds affected the metabolites or disrupted normal metabolic processes. Canola meal also contains significantly more choline compared to soybean meal, which may also influence lipid composition, namely the PC. Also, the two flax treatments contain significantly higher n-3 content, which will likely influence TG and PL composition, and could potentially impact cholesterol metabolism. Because TG and PL are present in all kinds of foods, different types of these lipids cannot be separated within each food product, making the determination of the metabolic pathway of each compound difficult to analyze (Küllenberg and others 2012). As a consequence, the health effects of such lipids are not clearly defined in the literature.   Heat map analysis (data not shown) showed that greater concentration of one of the two detected TG in addition to cerebroside A was found in treatments with flax compared to treatments without flax.

Cerebrosides are important components of animal muscle and nerve cell membranes. Whether or not consumption of eggs containing greater levels of this compound would result in a biological effect has yet to be determined. One study determined the cerebroside content in the brains of male weanling CFY rats after feeding 20% lipid from a variety of sources for 16 weeks (Srinivasarao and others 1997). The authors found that myelin membranes in the cerebra from rats fed groundnut or safflower oil contained significantly more cerebrosides compared to those fed coconut or mustard oil.

Little is known about the influence of the position of FA within dietary TG on lipoprotein concentrations and cholesterol metabolism. Triglycerides are absorbed in the intestine after hydrolysis to sn-2-monoglycerides and FA (Kayden, Senior, Mattson 1967), and are then resynthesized into TG and secreted in chylomicrons. The chylomicron TG largely retain their original fatty acid in the sn-2 position (Kayden, Senior, Mattson 1967; Mattson and Volpenhein 1964). Fatty acids attached to the sn-2 position might then be preferentially transported to the liver instead of to the extrahepatic organs because lipoprotein lipase, like pancreatic lipase, primarily attacks the sn-1 and sn-3 position of TG (Nilsson-Ehle and others 1973). Because the hepatocyte is the major site of action of FA on LDL metabolism, SFA in the sn-2 position of dietary TG might elevate LDL concentrations more than the same fatty acid in the sn-1 or sn-3 positions (Spady, Woollett, Dietschy 1993). Researchers found that SA present on all three positions of glycerol, in the form of a synthetic fat, raised cholesterol concentrations (McGandy, Hegsted, Myers 1970). In contrast, this effect was lessened when the SA was esterified to the sn-1 and sn-3 positions, in the form of cocoa fat. Extensive research has demonstrated the importance of consuming adequate n-3 PUFA and their relation to health and disease prevention,

but the role of specific TG configurations containing such PUFA, and different combinations of

PUFA are relatively unknown.

There are numerous types of PL found in egg yolk that perform related, but different

functions; a) lecithin or phosphatidylcholine (PC) is a choline reserve, and provides

diacylglyceride (DAG), FA and phosphorylcholine for cell signaling, b) PE plays a role

in membrane fusion and is primarily found in nervous tissue, c) sphingomyelin is a neural

membrane PL that also participates in cell signaling and, d) plasmalogen is found in sarcolemma,

the membrane of cardiac muscle.

Upon ingestion, PL are almost completely absorbed (> 90%) in the intestine (Küllenberg

and others 2012). Most of the PL are hydrolysed in the lumen at the sn-2 position by the

pancreatic phospholipase A2 and then taken up by the enterocytes as free FA and lysoPL

(Küllenberg and others 2012). Both can be re-esterified to PL and enter the bloodstream

incorporated in chylomicrons and, in a smaller proportion, VLDL. It has been shown that dietary

PL are able to deliver their FA for incorporation into cellular membranes, thus altering the

membrane composition of the cells. Consequently, cellular functions, including signaling and

transport, as well as the activity of membrane bound enzymes, could be modulated by dietary PL

and hence contribute to the observed health benefits. An *in vitro* study with hepatic cancer cell

lines showed a dose dependent growth restraint when the cancer cells were cultured in the

presence of egg yolk PC (Sakakima, Hayakawa, Nakao 2009). A possible explanation for this is

an increased lipid peroxidation rate as a consequence of structural and functional shifts in the

cellular membrane (Küllenberg and others 2012). It was reported that sphingomyelin had a

significant effect on blood cholesterol levels (Noh and Koo 2003; Noh and Koo 2004). The

authors suggested that this compound could produce a similar effect as phytosterols, which may

slow the rate of luminal lipolysis, micellar solubilisation and transfer of micellar lipids into the enterocytes. Another study showed that a supplementation with PL purified from egg yolk may improve learning abilities and visual function in age related impairment.

It is evident that further research needs to be conducted to determine the health benefits of specific TG and PL compositions containing n-3 PUFA. Additionally, without performing MS/MS on the compounds identified using LC-QTOF-MS in this study, it is unclear what the specific composition of the TG and PL are. However, this non-targeted approach has provided valuable insight into which general class of lipids can be elevated or decreased through dietary changes.

Conclusions

The industry may not have a true appreciation of the extent of changes in egg constituents when layer diets are manipulated. The objective of this research to develop a method using a non-targeted metabolomics approach to identify changes in yolk composition in response to alterations in the hen's diet was successful. Overall, it appears that the addition of CM and FO into the laying hen diets resulted in yolk metabolite changes. There is great potential to use yolk metabolomics in developing new functional eggs. Further studies using MS/MS and NMR would need to be conducted in order to validate the identity of these compounds. Additionally, the development of a database for egg yolk compounds will be useful for the future in designing tailored diets to target specific compounds.

Acknowledgment