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Production and Application of trans, trans CLA-rich Eggs: Chemical and Physiological properties and Prospects for Value-added Foods

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Production and Application of *trans, trans* CLA-rich Eggs: Chemical and Physiological properties and Prospects for Value-added Foods

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Food Science

by

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May 2016
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This thesis is approved for recommendation to the Graduate Council.

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ABSTRACT

Conjugated linoleic acid (CLA) is an 18-carbon fatty acid with conjugated double bonds that is naturally present in beef and dairy products. Certain CLA isomers exhibit human bioactivity such as anti-obesity and muscle metabolism effects, as well as prevention of age-associated conditions, and improved immune and inflammatory responses. However, an individual needs to consume 3-4 g CLA/day to realize these potential health benefits, and a Western diet only provides a tenth of this recommendation. Chicken eggs have been used as a commercialized vehicle to deliver other bioactive lipids such as omega-3 fatty acids, because the lipid profile of the egg yolk is easily modified through dietary inclusion. Therefore, eggs may also be suitable for delivering additional CLA to a person's diet so they may realize the potential health benefits. This program of study explored the use of a novel CLA-rich soy oil in poultry diets to produce CLA-rich eggs and determine their specific physiochemical properties. In addition, CLA-rich eggs were used to prepare CLA-rich mayonnaise and egg patties in order to determine the extent of CLA-rich egg use as a functional ingredient. The overall objectives were: 1) Describe efficient methodology for the analysis of egg yolk lipids. 2) Determine the CLA accumulation in eggs in layer hens, breeder hens and jungle fowl. 3) Determine the effect of CLA accumulation in egg yolk lipids on fatty acid composition of egg yolk triacylglycerides and phospholipids relative to those in conventional eggs. 4) Determine the effect of CLA in egg yolks on physical, rheological and egg quality properties. 5) Determine the functional effect of CLA rich eggs on the quality of processed food, and their physiochemical properties. This dissertation includes nine chapters on the novel findings from producing CLA-rich eggs. One CLA-rich egg can provide 140 mg additional CLA to the diet, a tablespoon of CLA-rich mayonnaise can provide 1.8 g of CLA, and a fried egg sandwich combining all of these ingredients can provide 2.8 g of CLA. Moreover, this research provided current egg literature with a comprehensive description of yolk lipid modifications that result from CLA incorporation, effects on egg quality, and use of CLA-rich eggs in food production.

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DEDICATION

To Nathan Shinn

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Mayfield, S., Proctor, A., **Shinn, S. E.**, Dewettinck, K., & Patel, A. R. (2015). CLA-rich soy oil shortening production and characterization. *Journal of the American Oil Chemists' Society*, 92(9), 1267-1275.

INTRODUCTION

Conjugated linoleic acid (CLA) is a fatty acid containing 18 carbons and a conjugated double bond configuration. This collection of positional and geometric isomers are products of ruminant fermentation, contributing less than 1% to total dairy fat or beef fat. Over 100 human studies have been published establishing strong evidence of CLA's effects on: anti-obesity, muscle metabolism, prevention of age-associated conditions, and improved immune and inflammatory responses (1). The majority of these studies used *cis*-9, *trans*-11 CLA isomer-dominated mixtures (1). While there is no Recommended Daily Allowance value for CLA, three meta-analyses have confirmed that CLA supplementation induces "modest but significant loss in body weight and body fat mass when CLA is supplemented at 3.2-3.4 g/d for at least six months" (1). CLA is *Generally recognized as Safe* (GRAS status) and this type of *trans*-fat is exempt from the recent FDA ban on partially hydrogenated trans fats. The Western diet does not provide sufficient CLA intake, at roughly 0.2 g/d. Therefore, it seems intuitively advantageous to develop low fat, low cholesterol, CLA-rich foods. This endeavor is currently viewed as a possible tactic to combat obesity and atherosclerosis in the United States (1).

Exposing soybean oil to UV radiation in the presence of an iodine catalyst produces a ~20% CLA-rich oil (2). Subsequently, the methods for CLA soy oil production improved (3), and the oils have been used to make margarine (4), shortening (5), and chocolate bars and pastes (6). Soy oil CLA differs from dairy, beef, and synthetically produced sources in that 1) the CLA is intact triacylglycerol form and 2) it contains primarily *trans, trans* CLA, amounting to approximately 70% of total CLA isomers.

Including CLA-rich soy oil in poultry feed will likely produce eggs with CLA-rich yolks, and a relatively high ratio of *trans, trans* isomers. This may provide a significant added value to the nutrition and functionality of the egg (3-8). Studies have reported superior bioactivity from *trans, trans* isomers relative to other CLA isomers, but have not yet been used in poultry studies (8,9).

Several studies report CLA enriched egg production by supplementing animal diets with commercially available CLA sources, which were almost entirely *cis*-9, *trans*-11 isomers in free fatty acid form (1). This resulted in efficient transfer of CLA into egg yolk, and dietary CLA doses are linearly related to egg CLA concentration. Unfortunately, previous studies have also reported adverse effects on the quality of the eggs, including: development of rubbery yolks and pink albumen, and unhatchable eggs (9,10).

However, the addition of 6% soy oil to a CLA-enriched diet restored laying to 100%.

This doctoral dissertation investigates soy oil CLA accumulation in chicken eggs. It explores the structural chemistry of the CLA-rich yolk lipids, their physiochemical properties, and their functionality in food products relative to conventional eggs. The overall objectives were:

1. Describe efficient methodology for the analysis of egg yolk lipids.
2. Determine the CLA accumulation in eggs in layer hens, breeder hens and jungle fowl.
3. Determine the effect of CLA accumulation in egg yolk lipids on fatty acid composition of egg yolk triacylglycerides and phospholipids relative to those in conventional eggs.
4. Determine the effect of CLA in egg yolks on physical, rheological and egg quality properties.
5. Determine the functional effect of CLA rich eggs on the quality of processed food, and their physiochemical properties.

I hope that this document provides the reader with a logical, concise collection of published manuscripts on CLA egg enrichment, egg analysis, and egg application. Furthermore I hope that it conveys how elegant and thoughtful a Food Science doctoral program can be, if given hard-work and dedication. And a great advisor. Wink.

Sara Shinn

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CHAPTER 1. Egg yolk as means for providing essential and beneficial fatty acids

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Abstract

Chicken eggs are an elective vehicle for delivering essential, beneficial fatty acids to a person's diet, as yolk lipids can be enhanced by incorporating specific lipid sources into the bird's diet. Poultry feed rich in omega-3 fatty acids (O3FA) has allowed commercialization of enriched eggs containing up to 600 mg of O3FA. Dietary O3FA prevents cardiovascular disease (CVD), and aids brain structure and function. Conjugated linoleic acid (CLA) has also been used to enrich eggs. CLA consumed at 3 – 4 g/ day promotes weight loss to combat obesity. However, Americans consume only 50% of the O3FA recommended adequate intake, and average CLA consumption is under 600 mg/day. While a variety of foods are naturally rich in O3FA, conventional sources of CLA are limited to bovine milk and meat, which do not provide enough CLA to produce clinical effects in a balanced diet. The new Dietary Guidelines suggest that a person's diet should optimize the types of fat consumed, instead of reducing or eliminating it from the diet. Since eggs and egg-based products are common in the Western diet, eggs enriched with O3FA and CLA may increase consumption to the recommended levels. This article discusses 1) health benefits of O3FA and CLA; 2) the design of nutritionally enhanced eggs; and 3) the future direction of enriched egg research.

Introduction

Obesity and cardiovascular disease (CVD) are major public health concerns affecting all segments of the population [1]. The previous version of the Dietary Guidelines on fat consumption was established in 1980 and recommended that Americans consume less than 30% of their total calories from fat [2]. For the past 35 years Americans have complied with these guidelines by reducing their consumption of eggs, full-fat dairy, and red meat in an attempt to adopt a "heart-healthy" lifestyle [3, 4]. However, obesity and heart disease continue to be serious health concerns for Americans, despite the reported changes in dietary fat intake. Furthermore, recent meta-analyses conclude that there is not sufficient evidence that reducing total fat and/or saturated fat intake decreases death rates from heart disease [5, 6].

With the intent to inform Americans how they can improve their overall eating patterns, the newly released Dietary Guidelines for Americans have updated their recommendations on fat intake [7]. These new guidelines no longer include an upper limit for fat intake, and cholesterol is no longer considered a

nutrient of concern [7]. This is because evaluation of the scientific literature indicates that there is no relationship between dietary cholesterol intake and serum cholesterol levels [6]. Additionally, randomized, controlled trials demonstrate that diets higher in nutraceutical fats, such as omega-3 fatty acids (O3FA) and conjugated linoleic acid (CLA), reduce the risk of CVD [8]. Furthermore, recent data demonstrate that regular consumption of eggs does not alter cholesterol levels, and can actually improve post-meal metabolic responses [25-35]. Therefore, it is important to understand the nutritional value of egg yolks enriched with O3FA and CLA, and how they may help optimize the fats provided in a typical American diet. This article communicates 1) the health benefits of O3FA and CLA; 2) the design of nutritionally enhanced eggs to realize these benefits; and 3) the future direction of enriched egg research.

Omega-3 fatty acids in human health

Foods that are major contributors of O3FA include fish, other seafood, chicken, eggs, as well as canola, soy and flaxseed oils. Legumes, green vegetables, cauliflower, whole milk and ground beef provide small quantities of O3FA. The simplest O3FA is alpha-linolenic acid (ALA). Other O3FA fatty acids of this family are derivatives of ALA with longer, more unsaturated hydrocarbon chains, including eicosapentaenoic acid (EPA, 20:5(n-3)) and docosahexanoic acid, (DHA, 22:6(n-3)). Figure 1 contains simple structural formulas for the mentioned O3FA.

ALA is most often recognized for its conversion to DHA and EPA in the body [11]. Recently, the European Food Safety Authority confirmed a cause and effect relationship between the dietary intake of ALA and brain and nerve tissue development [12]. DHA is the O3FA fatty acid that is internationally recognized as playing a crucial role in brain development and function [10, 11, 13]. DHA provides the correct physical conditions for signaling receptors, transporters, and other membrane proteins to function [10, 11]. New evidence names DHA as a precursor for powerful antioxidant neuroprotectins, which would explain a mechanism for brain protection against oxidative stress [14]. DHA deficiencies contribute to brain maintenance problems and oxidative stress, which is considered a contributor to heart disease, as well as Alzheimer's disease [13, 15]. EPA is important in blood flow and control of inflammation [11]. Similar to DHA, levels of EPA are primarily determined by dietary sources, although limited EPA synthesis

from ALA sources occurs in adults [56]. In experimental and animal models, both EPA and DHA modify numerous biologic pathways, with evidence for some differential benefits against CVD [59].

Evidence of O3FA health benefits has repeatedly shown CVD prevention [9-11, 56-59].

Governmental and international administrations have established minimum consumption levels of O3FA [57, 58]. The different O3FA guidelines usually provide a combined EPA+DHA recommended intake, and are based on primary prevention of CVD. Nutrition guidelines generally recommend a minimum consumption of 250–500 mg/d of combined EPA+DHA. Unfortunately, most Western diets only contain 50% of the ALA recommended adequate intake, which is 1.6 g/day for men, and 1.1 g/day for women [8]. In addition, average EPA + DHA intakes in most countries, including in the US are much lower than the recommended amounts [60].

Furthermore, the dietary source of O3FA has been recently emphasized as important. While direct consumption of an O3FA-rich food has been deemed beneficial for the prevention of CVD [61, 62], the efficacy of commercially available O3FA supplements on the prevention of CVD has been recently deemed inconclusive [63]. However, the present evidence suggests that the O3FA naturally present in fish and eggs share and complement each other's nutritional benefits, and increasing human ingestion of O3FA would be advantageous to public health in the prevention of CVD, when compared to little or no consumption.

Finally, the ratio of omega-3 and omega-6 fatty acids in the diet is also an important dietary consideration [81]. While American diets are considered deficient in O3FA, they consume excessive amounts of omega-6 fatty acids, and the ratio of omega-6: omega-3 in the American diet has been cited as high as 16.7:1 [81]. The disproportion of these two types of fatty acids promote the pathogenesis of many diseases, including CVD, while a low omega-6: omega-3 ratio (~1) in the diet exerts suppressive effects. In the secondary prevention of cardiovascular disease, a ratio of 4:1 was associated with a 70% decrease in total mortality [82]. Therefore, a lower ratio of omega-6/omega-3 fatty acids is more desirable in reducing the risk of many of the chronic diseases of high prevalence in Western societies.

Conjugated linoleic fatty acids (CLA)

CLA is an 18-carbon fatty acid with 2 conjugated double bonds that is in beef and dairy products. Presently, CLA is available as commercial supplements, or is provided in limited amounts in beef (0.2–10.0 mg/g lipid) and dairy products (0.2 – 3.3 mg/g lipid) [64, 65]. Literature on CLA and health benefits recommends 3 – 4 g of CLA a day to achieve potential health benefits, but conventional beef and dairy sources do not provide sufficient CLA to produce a clinical effect. Estimates of the average U.S. consumption are approximately 200 mg CLA / day for men and 90 -150 mg CLA/ day for women [66].

There are a variety of CLA positional and geometrical isomers containing double bonds at various conjugated positions. The most naturally prevalent CLA isomer is *cis*-9, *trans*-11 CLA [67]. When CLA is produced from linoleic acid using chemical processes, a significant amount of *trans*-10, *cis*-12 CLA, as well as some *trans*, *trans* CLA are formed along with the *cis*-9, *trans*-11 isomer [47,68]. Linoleic acid and the three mentioned CLA isomers are presented in Figure 2.

There are a number of human studies pairing the effect of CLA on body fat and body composition [20, 21, 68-70], as well as CLA and CVD [19-21]. The *trans*-10, *cis*-12 CLA isomer has been deemed responsible for body fat reduction [20, 25, 27]. Three meta-analyses of human CLA studies have established that CLA does induce significant body weight loss with 3.2-3.4 g/ day CLA supplementation for at least 6 months [21, 69, 70]. More recently *trans*, *trans* CLA has been recognized for isomer-specific bioactivities in animal models [16-19, 71]. Furthermore, CLA is effective in reducing fat gain during a weight gain period [25]. CLA's effect on body fat reduction is suggested to be the result of multiple effects, including increasing energy expenditure and reducing lipid accumulation in adipose tissues [20, 26]. However, applying CLA in human nutrition to combat obesity necessitates further studies to determine the most effective strategies.

Reports of direct consequences of CLA on CVD have been less promising. CLA studies of CVD indicators often report no changes in cholesterol, LDL, triacylglycerols or non-esterified fatty acids. While some publications have reported that CLA decreased blood pressure [21-24], a recent meta-analysis concluded that CLA has no positive effects on human blood pressure [72]. However, two studies reported that CLA supplementation along with calcium decreased pregnancy-induced hypertension, increased birth-weights, and improved endothelial function without affecting blood pressure parameters [22]. While

CLA has not been shown to have a direct preventative effect on CVD, it does have nutritional merit and potential to improve other health issues, particularly with regard to symptoms of menopause, bone health sarcopenia and sarcopenic obesity [68], and the potential of this fatty acid needs to continue to be explored.

Eggs enhanced nutritionally and functionally by fatty acids

The advice to avoid excess egg consumption in order to avoid increased serum cholesterol is now out of date [7] and more recent data demonstrates that regular consumption of eggs does not alter cholesterol levels, and can actually improve post-meal metabolic response [25-35]. In addition, eggs are a good source of protein, choline, vitamin D, lutein and zeaxanthin. Besides their nutritive value, eggs as a food ingredient provide versatile functional properties in prepared foods ranging from baked items, confectionaries, ice cream, mayonnaise, salad dressings, and pasta. Furthermore, it has been shown that egg yolk lipid composition can be manipulated to substantially increase concentrations of specific fatty acids, as yolk lipids are closely linked to the hen's dietary lipids [34, 35]. This makes eggs a useful vehicle for delivery essential and beneficial fatty acids. With the high prevalence of obesity and CVD, nutritionally enhanced eggs could play a larger role in promoting the nutritive value of US diets.

Omega-3 eggs: Eggs natively contain 50 – 60 mg of O3FA /100 g of egg, and are already considered a valuable source of O3FA in western diets. Plant and marine O3FA incorporated into laying hen diets have resulted in accumulation of ALA, DHA, and EPA in table eggs [34-36]. By feeding chickens linseed, for example, O3FA enriched eggs have approximately 20-fold greater ALA levels, and feeding fish oil results in 6-fold greater DHA, relative to standard control eggs [36]. In addition, O3FA eggs have up to a 2-fold reduction in omega-6: omega-3 ratio [34]. Furthermore, these eggs have significantly greater PUFA: SFA ratio [34-36].

In an O3FA egg and human hypercholesterolemia trial, 28 men each ate 4 O3FA eggs per day, and there was no significant difference in their total cholesterol, HDL cholesterol, or total plasma triglycerides, relative to when subjects were consuming diet without eggs [37]. Moreover, after eating O3FA eggs, the subjects had greater blood plasma ALA and DHA concentrations and a lower omega-6: omega-3 ratio in their platelet phospholipids, relative to when their diet included control eggs, or was eggless. Also, statin-

treated hypercholesterolemic patients consuming O3FA eggs exhibited a 23% rise in EPA and DHA levels in serum phospholipids, which relates to a reduced risk of CVD [38]. O3FA in eggs is a promising dietary constituent that complies with nutritional recommendations for chickens, and the daily consumption of one egg enriched in O3FA can compensate for 30% of the recommended adequate intake for men, and 48% for women [35].

The importance of O3FA during pregnancy was recently reviewed, and showed three main benefits: positive effects on fetal and neonatal development, preterm birth, and perinatal depression [35]. Egg lecithin has been precipitated from O3FA enriched yolks and added to several brands of baby formula, so that these formulas provides the same quantities of O3FA FA as mother's milk and the blood plasma and cells fatty acid composition of babies fed the O3FA FA enriched formula were identical to those of breast-fed babies [35].

The sensory challenges of O3FA eggs have been pronounced fishy aromas and flavors when hens are supplemented with >5% fish oil, but the strategy has been the addition of antioxidants, or microencapsulation of the fish oil to reduce oxidation in the prepared diets [35,79]. O3FA enrichment has not changed the functionality of eggs, with the exception that O3FA eggs produced from flaxseed supplementation exhibit less foaming capabilities relative to eggs supplemented with other O3FA sources. [35,80]. The food functionality of O3FA eggs, including emulsification capacity, hardness and springiness of sponge cakes prepared using these eggs, were the same as conventional eggs [39]. However, information on O3FA egg functionality in different egg-rich products is lacking. Data on effects of O3FA enrichment on egg quality during storage have determined that increase O3FA does not decrease oxidative stability in stored shelled eggs [73,74] However, O3FA eggs do develop weaker vitelline membranes than control eggs after refrigeration [40]. Further determinations on the shelf-stability and functionality of O3FA eggs are needed.

CLA-rich eggs: CLA-enriched eggs have been produced and studied extensively in a research environment, but are not commercially produced. Eggs produced by hens fed 5.0% CLA can contain 310–365 mg of CLA per egg [41-43]. Early CLA-rich egg production studies concluded that CLA egg production was not practical because adding more than 4% of CLA fatty acids resulted in reduced egg quality by yolk and albumen weight reduction [45, 46]. In all previous studies, CLA egg enrichment

induced a 34% increase in yolk saturated fatty acids, and decreased monounsaturated fatty acids (MUFA). However, adverse effects on egg quality were reduced or prevented when CLA was co-supplemented with other oils, such as olive, canola, or soy oil [46, 75].

Soy oil has been used to produce CLA-rich triacylglycerols [47, 48]. A subsequent study feeding 4% of this CLA-rich soy oil to obese Zucker rats reduced serum cholesterol by 38% and LDL cholesterol by 50%, relative to the control obese rats fed 4% conventional soy oil for 100 days [18]. In subsequent egg enrichment studies, CLA-rich soy oil was fed to chickens to produce CLA-rich yolks containing 115-150 mg CLA per egg [49,50].

When CLA-rich soy oil was fed as 1.5% of chicken diets, *trans, trans* CLA isomers were the most abundant in the yolk, followed by *trans*-10, *cis*-12 CLA [49]. A later study determining CLA-rich soy oil's effects on hen egg quality showed that eggs obtained with the CLA-rich soy oil diet were similar in size to the non-CLA controls and saturated fat was increased by only 28% (relative to 34% in other studies) [41]. Furthermore, the CLA-rich yolks were significantly more viscous than control yolks [50]. These findings were in contrast to previous CLA egg enrichment studies with *cis, trans* CLA mixtures, which produced smaller yolks and up to a 34% increase in yolk saturated fatty acids [46].

On finding that CLA promoted yolk viscosity, these eggs were used to enhance mayonnaise viscosity [51]. CLA-enriched eggs increased mayonnaise emulsion stability, reduced oil droplet size, increased mayonnaise thickness and viscosity, retained its rigidity longer, and resisted over-spreading and thinning, relative to control yolk mayonnaise formulations that used conventional eggs. A mayonnaise prepared with both CLA-eggs and CLA-rich soy oil can provide up to 1.8 g of CLA per tablespoon, which is more than half of the suggested amount of CLA needed to produce clinical effects. The study showed that simply manipulating egg yolk fatty acid composition has a significant effect on food functionality, and more studies are needed on egg functionality in relation to its lipid composition.

A recent study showed that dietary CLA-rich egg powder increased CLA in the liver, splenocytes, and plasma in rat and hamster models, relative to rodents fed control egg yolks [35, 52]. The significant increase of CLA in tissue incorporation, antioxidant activity, and fecal lipid and cholesterol excretion suggest that egg yolk CLA has a beneficial effect on lipid and cholesterol metabolism. Determining the effect of CLA-rich egg triacylglycerol and phospholipids on human health would be very helpful in

understanding the potential benefits of this product. If CLA-rich eggs can provide a substantial amount of CLA to human diets, this functional food not only would provide a nutritious ingredient that is accepted by much of the Western population, and it may play a role in combatting obesity and CVD.

O3FA and CLA on egg yolk fatty acid composition: The incorporation of O3FA versus CLA into egg yolks have very different effects on total yolk lipid fatty acid (FA) composition, as well as the FA composition of the triacylglycerols versus the phospholipids present in the yolk. In a previous study from our research group, the FA composition of CLA-rich triacylglycerols and phospholipids were reported as an attempt to provide the egg lipid literature with a more complete understanding of how dietary CLA inclusion affects egg yolk lipid composition [83]. Here we provide additional data on the FA profiles of O3FA-enriched eggs that are commercially available and state to contain “225 mg of omega-3 in each egg” (Organic Valley Omega 3 Large Grade A Brown Eggs). Figure 3 contains a summary of the total FA composition, as well as the triacylglycerol and phospholipid FA composition of CLA-rich, O3FA-rich, and standard control egg yolks.

Figure 3a shows the fatty acid composition for total lipid extractions from each type of egg. Statistically significant differences were defined at the 0.05 α -level. CLA eggs contain 43.1% SFA, which is significantly higher than commercial table and omega-3 eggs at 36.2% and 36.0% SFA. PUFA concentrations in CLA yolk was also significantly lower in comparison to both commercial egg types. CLA egg yolk MUFA concentration was 31.5%, which was significantly lower than both commercial egg types. PUFA concentration was highest in CLA eggs at 24.5%, followed by omega-3 yolks at 22% and control eggs at 14.0%. Long chain PUFA (LC-PUFA) concentrations were highest in omega-3 yolks at 1.7%, followed by control yolks at 1.4% and CLA yolks at 0.8%.

Figure 3b presents the triacylglycerol (TAG) fatty acid concentration for control, CLA-rich, and O3FA yolk, grouped by degree of unsaturation. The relative proportions of fatty acid groups closely resemble what was seen in total lipid fatty acid composition. SFA concentration in CLA yolk TAGs was 46.9%, which is significantly higher than control commercial yolk at 37.7% and omega-3 yolk TAG at 31.8%. CLA yolk TAGs had significantly lower MUFA concentration at 35.4%, while control yolk TAGs had 45%. PUFA concentration was also highest in omega-3 yolk TAGs at 23.9%, while CLA TAGs had 17.4% PUFA. Regular eggs yolks contained the highest levels of LC-PUFA in TAG fraction at 3.6%, relative to CLA yolk

at 0.2% and omega-3 0.7%. This is despite the advertised higher omega-3 and DHA content on commercial omega-3 eggs.

Figure 3c depicts the phospholipid (PL) fatty acid composition for control, CLA-rich, and O3FA-rich yolk. CLA yolk PL total SFA concentration was 45.8%, significantly higher than control yolk PL at 42.6%. Control yolk had significantly less SFA. The amount of MUFA in standard control yolk PL is significantly greater than the enriched eggs at 59%. However, total MUFA and PUFA concentrations were not significantly different between CLA and omega-3 eggs. In contrast to the TAG fraction, the PL FA compositions of CLA and O3FA, are very similar to each other while standard control yolks are drastically different from the enriched eggs.

In summary, O3FA enrichment has very different effects on the total lipid composition of egg yolks relative to CLA enrichment, and these differences are further distinguished by the specific lipid fraction. In the TAG fraction, O3FA more closely resembles standard control eggs, while in the PL fraction the CLA and O3FA profiles are more similar. Determining the correct amounts of CLA and O3FA to enrich an egg in both beneficial fatty acids, without significantly increasing saturated fat would maximize the nutritive value of the egg yolk lipids would be ideal, and attempts to do so are discussed next.

Dual O3FA and CLA eggs: There have been a limited amount of studies that have enriched eggs using both O3FA and CLA supplementation in the hen's diet [76-78]. Co-supplementation did not significantly lessen the effects of CLA enrichment on fatty acid composition, such as increased saturated fat levels. Co-supplementation did however, reduce volatiles associated with fishy off-flavors that are prevalent in some O3FA rich eggs. It has been shown that other vegetable oils supplemented with CLA do lessen the fatty acid composition modifications associated with CLA inclusion [46, 75]. However, expanding research on the combination of dietary lipids that: 1) increase both O3FA and CLA egg content 2) do not significantly increase saturated fat content and 3) are absent of any organoleptic challenges and 4) have similar or enhanced functional properties and oxidative stability relative to control eggs be beneficial to the poultry feed industry and consumers wanting to increase their healthy lipid intake.

Conclusions

Recent evidence has toted the beneficial effects of high-density lipoprotein cholesterol and triacylglycerols, and randomized human trials have confirmed that diets containing greater amounts of healthy fats, like vegetable oils and fish, along with greater protein amounts, and reduced refined carbohydrates, reduce the risk of CVD [53-55]. Furthermore, the new US dietary guidelines have withdrawn advice on total fat limits and removed dietary cholesterol as a "nutrient of concern" [1]. While it has been demonstrated that levels of O3FA in an egg can be increased 20-fold by the inclusion of plant and marine O3FA in the chicken diet, the availability of O3FA enriched eggs is still limited [6, 25, 26]. However, increasing the availability and consumption of these eggs may be beneficial in combatting CVD. Likewise, CLA-rich eggs may also be a diet-based approach to preventing or ameliorating the progression of CVD by combatting obesity. O3FA and CLA consumption in Western diets may be increased by providing access to eggs enriched with one or a combination of beneficial fatty acids, or by utilizing these eggs as ingredients in other foods such as mayonnaise, pasta, salad dressings, or baked goods. However, the success of enriched eggs and egg products will depend upon economic feasibility, acceptable sensory characteristics and stability during cooking, storage and processing, and need to be investigated in detail.

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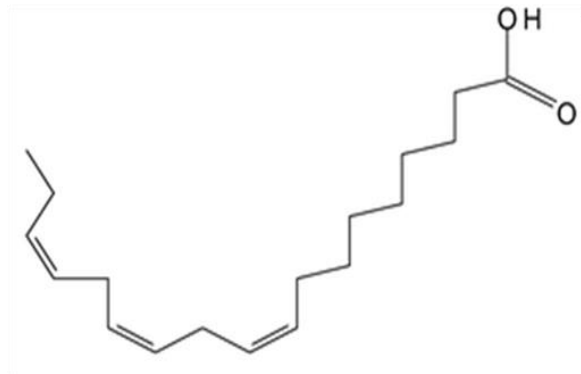
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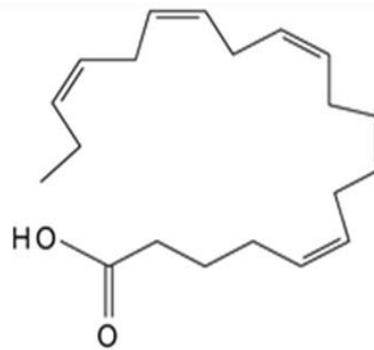
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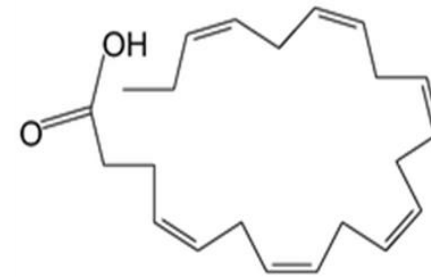
Figure 1. The omega-3 fatty acids: alpha- linolenic acid (ALA, 18:3(n-3), 18:3 ω 3); eicosapentaenoic acid (EPA, 20:5(n-3), 20:5 ω 3); and docosahexanoic acid, (DHA, 22:6(n-3), 22:6 ω 3).



ALA

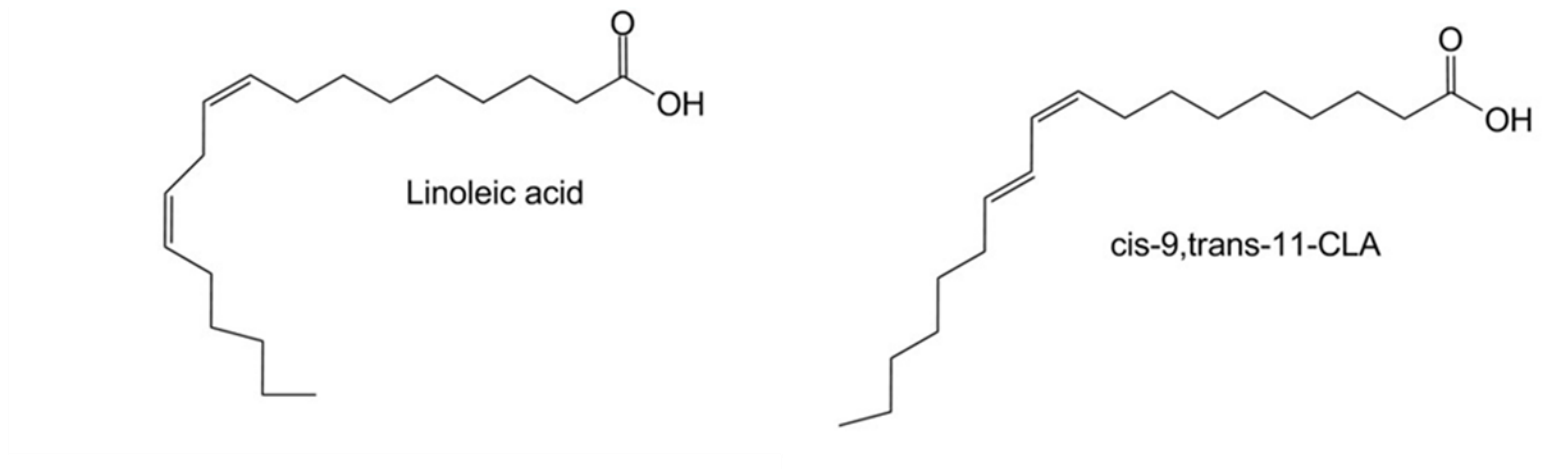


EPA



DHA

Figure 2. Linoleic acid and conjugated linoleic acid (CLA) in three isomeric forms: cis-9, trans-11, trans-10, cis--12, and trans-9, trans-11 CLA.



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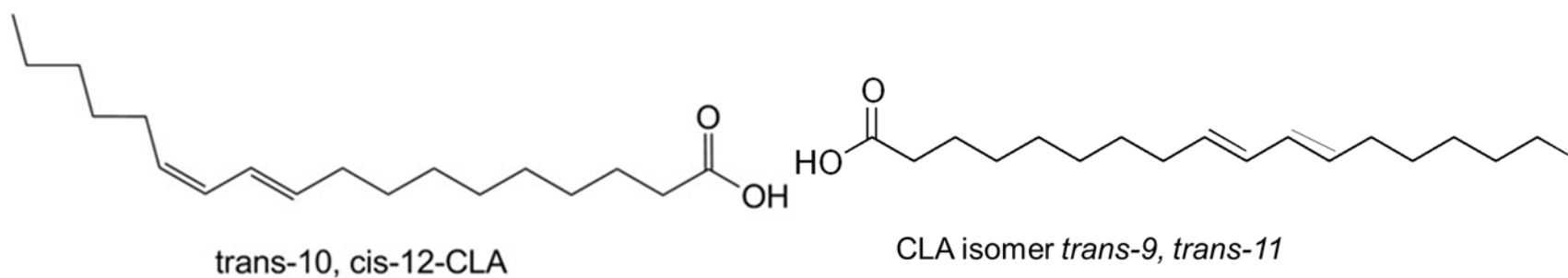
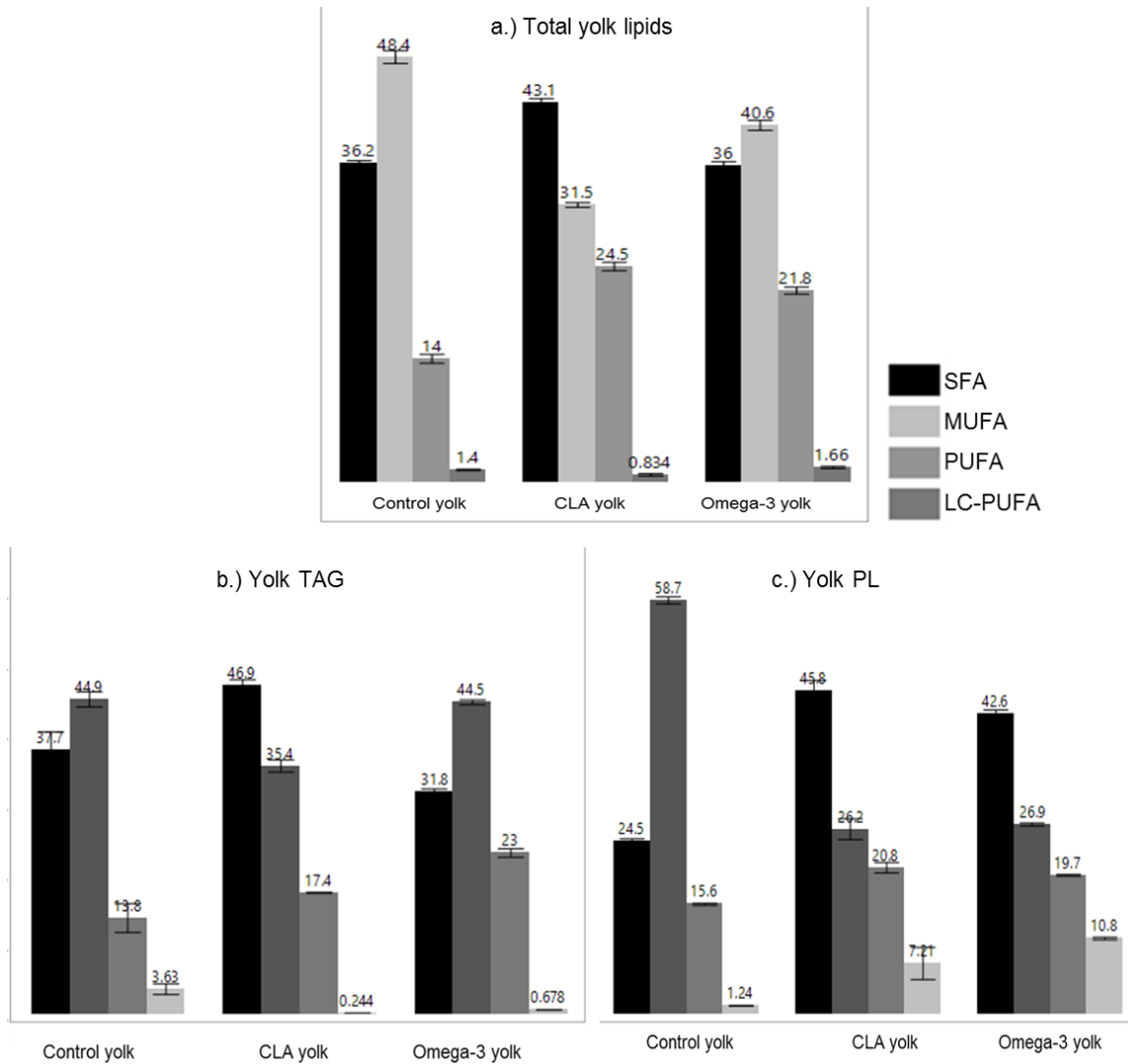


Figure 3. Fatty acid composition of a.) total yolk lipids b.) yolk triacylglycerols (TAG) and c.) Yolk phospholipids (PL) extracted from CLA-rich, omega-3 rich, and standard control egg yolks. Fatty acids are grouped by degree of unsaturation: saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), long chain polyunsaturated fatty acids (LC-PUFA).



CHAPTER 2. LETTER TO THE EDITOR: Rapid Lipid Extraction from Egg Yolks

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Sir,

Total or crude fat determination is commonly measured in lipid chemistry by traditional methods such as Soxhlet or Goldfisch extraction. These methods require hours of solvent reflux extraction, which may be necessary when lipids are well embedded into tissues and the surface area to weight ratio is low.

However, many food materials may have oil that can be readily extracted without such exhaustive solvent extraction. Simple, practical, rapid extraction techniques are more cost effective when dealing with large number of samples if the lipids can be readily extracted from the food matrix.

Clark and Snyder [1] developed a rapid, 1-min equilibrium extraction method of a 1–2-g soy flour sample with hexane at ambient temperature that produced results similar to those obtained by Goldfisch extraction to allow rapid screening of soybean cultivars. Subsequently, we developed a rapid, 1-min method for total lipid extraction with hexane and isopropanol of 2 g of rice bran samples for free fatty acid determination that produced the same results as the Goldfisch extraction [2]. Similarly, the oil contents of milled rice and potato chips were determined by rapid solvent extraction [3, 4].

However, we have not developed any rapid animal lipid extraction methods. We anticipate incorporating CLA-rich oil [5] in eggs through poultry feed. Therefore, a rapid egg yolk lipid extraction technique would be invaluable prior to egg fatty acid analysis by FAMES GC-FID analysis. The Folch method [6] is a commonly used procedure for total lipid extraction from eggs, but uses chloroform–methanol (2:1, v/v) to extract the lipids, followed by a water wash using 0.2 times the volume of sample. This method is both time consuming and constitutes a significant health hazard because of the use of chloroform. The development of a practical, simple, rapid extraction technique would be more cost effective, particularly when dealing with a large number of samples. Hara and Radin [7] described an efficient 1-min extraction procedure that is particularly adapted to nervous tissues using hexane/isopropanol (3:2, v/v). Using this method, the whole liquid phase is evaporated, eliminating a phase separation step. The objective of this study was to determine if a rapid hexane/isopropanol extraction would extract the same amount of lipid and have the same fatty acid profile as lipids obtained by the Folch method.

Six eggs were collected from the University of Arkansas Poultry Science Department. Egg yolks were separated, combined and well mixed in a beaker with a stir-bar. Yolk samples were then diluted with distilled water to obtain 100, 75, 50 and 25 % dilutions of the original yolk mixture. Duplicate extractions of each dilution were made by the Folch method [6] and the following rapid extraction method. Duplicate 4-g samples were accurately weighed and vortexed with ten times the volume of hexane/isopropanol (1:1, v/v) for 5 min at room temperature. Homogenate was filtered using a funnel with Whatman no. 4 110-mm filter paper to recover only the liquid phase. Samples from both extraction methods were evaporated under vacuum in a rotary evaporator and weighed. A calibration curve was prepared comparing total lipid extraction by both methods. One-way ANOVA was determined by JMP 9.0.2 software to observe significant differences in the extraction methods.

Each duplicate extraction from the whole yolk and 50 % dilution was subject to duplicate GC-FID FAMES analysis. The FAMES were prepared using a rapid, micro-FAMES method [8], and FAMES were analyzed by GC-FID by the method of Christie et al. [9]. One-way ANOVA was determined by JMP 9.0.2 software to observe any significant differences in the fatty acid content obtained by Folch and rapid hexane/isopropanol extraction methods.

Table 1 shows the comparison of total oil obtained by each extraction method from each yolk dilution. The data show that there is no significant difference between the extraction methods at any specific dilution. Furthermore, the correlation between the two extraction methods shows a correlation coefficient (R^2) of 0.997 with an intercept of 1.08. The fact that the regression line does not go through the origin may mean that hexane-isopropanol extracted more non-lipids than the Folch solvent. This could be due not including a water wash in the hexane–isopropanol extraction. However, this method does not claim to exclusively extract lipids, but that non-lipid extracted do not affect the data relative to lipid determination by the Folch method.

Table 2 shows fatty acid composition of lipid extractions from both the Folch method and rapid hexane/isopropanol extraction methods. No statistically significant differences were found in the levels of each fatty acid present in yolk lipids, which reflects the egg lipid profile previously reported [10].

In summary, we developed a rapid extraction method for egg lipids that is an effective alternative to the Folch method. This method also provides accurate fatty acid profiles when compared to those obtained using a Folch extraction and subsequent GC-FID FAMES analysis.

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Tables and Figures

Table 1. Oil extracted from duplicate 4-g yolk dilutions by a rapid hexane/isopropanol extraction, relative to a control Folch extraction [6].

Yolk concentration (%)	Mean oil extracted (g) Folch method	Mean oil extracted (g) hexane/isopropanol method	Prob > F
25	7.65 ± 0.15 ^a	8.35 ± 0.35	0.29
50	16.2 ± 1.0	16.75 ± 0.35	0.40
75	22.95 ± 0.15	21.9 ± 0.41	0.13
100	37.35 ± 0.05	36.5 ± 0.32	0.21

Means are expressed with standard error of mean

^a Extracted oil amounts in the same row are not statistically different, shown by the large *P* values

Table 2. Fatty acid composition of whole egg yolk and 50% egg yolk dilution obtained by Folch and rapid hexane/isopropanol extraction methods.

Extraction method	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:4
Undiluted yolk								
Folch	0.37 ± 0.003a	25.5 ± 0.20b	3.0 ± 0.08c	8.9 ± 0.21d	40.6 ± 0.20e	16.5 ± 0.01f	0.33 ± 0.03g	2.3 ± 0.13h
Hexane/IPA	0.38 ± 0.003a	25.6 ± 0.20b	3.0 ± 0.08c	8.5 ± 0.21d	40.5 ± 0.20e	16.6 ± 0.01f	0.33 ± 0.03g	2.3 ± 0.13h
50 % Yolk dilution								
Folch	0.37 ± 0.003a	25.5 ± 0.20b	3.0 ± 0.08c	8.6 ± 0.16d	40.7 ± 0.06e	16.5 ± 0.01f	0.35 ± 0.02g	2.4 ± 0.23h
Hexane/IPA	0.38 ± 0.003a	25.6 ± 0.20b	3.0 ± 0.08c	8.5 ± 0.16d	40.5 ± 0.06e	16.6 ± 0.01f	0.34 ± 0.03g	2.3 ± 0.23h

Fatty acid percentages with the same letter in the same column are not statistically different

Means are expressed with the standard error of mean

CHAPTER 3. Improved fatty acid analysis of conjugated linoleic acid-rich egg yolk triacylglycerols and phospholipid species.

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Abstract

Reports from chicken conjugated linoleic acid (CLA) feeding trials are limited to yolk total fatty acid composition, which consistently described increased saturated fatty acids and decreased monounsaturated fatty acids. However, information on CLA triacylglycerol (TAG) and phospholipid (PL) species is unavailable. This study determined fatty acid (FA) composition of total lipids in CLA rich egg yolk produced with CLA-rich soy oil, relative to control yolks using GC-FID, determined TAG and PL FA compositions by TLC-GC-FID, identified intact PL and TAG species by TLC-MALDI-MS, and determined the composition of TAG and PL species in CLA and control yolks by Direct Infusion ESI-MS. In total, two lyso-phosphatidyl choline (LPC) species, one sphingomyelin (SM) specie, 17 phosphatidyl choline (PC) species, 19 TAG species and 9 phosphatidyl ethanolamine (PE) species were identified. Fifty percent of CLA was found in TAG, occurring predominantly in C52:5 and C52:4 TAG species. CLA-rich yolks contained significantly more LPC than did control eggs. Comprehensive lipid profiling may provide insight on relationships between lipid composition and the functional properties of CLA-rich eggs.

Keywords: *trans, trans* conjugated linoleic acid, egg yolk, fatty acids, triacylglycerols, phospholipids, GC-FID, MALDI-TOF-MS, direct infusion ESI-MS.

Introduction

Several conjugated linoleic acid (CLA) nutrition studies have reported anti-carcinogenic, anti-atherogenic properties, ability to increase lean body weight, and protect against immune-induced body wasting disease, and chronic inflammatory diseases (1, 2). Human clinical trials show that CLA was found to significantly decrease body fat (3), and waist size (4). In addition, the health effects of CLA seem to be isomer specific. For example, the *trans*-10, *cis*-12 CLA isomer is the more potent anti-obesity agent in mice relative to other isomers (5).

A novel CLA-rich soy oil has been produced by UV photoisomerization of soy oil linoleic acid, which produces triacylglycerols in soy oil with up to 20% CLA (6). Approximately 70 % of total CLA in CLARSO are *trans*, *trans* isomers, while the remaining are *cis*, *trans* and *trans*, *cis* isomers (7). *Trans*, *trans* CLA-rich soy oil effectively lowered serum cholesterol low density lipoprotein-cholesterol levels and liver lipid content in genetically obese rats (8). The *trans*, *trans* isomers accelerated apoptosis in vitro human gastrointestinal cancer (9). *Trans*, *trans* CLA showed greater inhibition of MCF-7 breast cancer cells, compared to *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA isomers (10). *Trans*, *trans* isomers also decreased atherogenesis-related genes in human umbilical vein endothelial cells and altered macrophage adhesion (11).

Research has shown that poultry feed enriched with CLA could be used to produce CLA-rich eggs with a potential market success comparable to that of omega-3 enriched eggs (12-17) as the fatty acid composition of chicken egg yolks is easily modified by dietary fatty acids (18). Previous dietary studies focused primarily on the changes in fatty acid composition, but rarely report complete fatty acid composition of triacylglycerols (TAG) and phospholipid (PL) species (19). Composition of TAG and PL species in conjunction with fatty acid composition of CLA-rich egg yolks would provide a more complete description of the deposition and the nutritional significance of CLA-rich soy oil in poultry diets.

Rapid Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF/MS) characterization methods have been used to qualitatively determine fatty acid composition of specific lipids, such as TAG and PL, in crude samples. Yolk lipids have a combination of TAG and PL, but direct MALDI-TOF/MS biased the detection of PL species over TAG (22). Decreased TAG detection is a result

of suppression by phosphatidylcholine (PC) (20-22). Fuchs et al. (23) reported an alternative method which couples TLC with MALDI-TOF/MS to avoid suppression of other lipid classes by PC. However, this method only described the egg yolk PL and disregarded the detection of the more abundant TAG. Lay et al. (24) addressed suppression by applying solid phase extraction (SPE) to separate TAG and PL species from beef and yolk lipid samples that could be then be analyzed by direct MALDI-TOF/MS analysis. However, analytical replications using this approach were found to be extremely difficult due to inherent heterogeneity in matrix crystallization in the MALDI preparation.

Direct infusion Electro-spray Ionization (ESI-MS) is an alternative method which has been used for PL and TAG lipid identification and characterization. However, only free fatty acids and small PL molecules are analyzed, while major TAG species remain undetected (25). Also, lipids that have different molecular elemental compositions may actually yield ions with the same ionic elemental compositions, depending on the ionic adduct that is formed upon ESI-MS. To solve this problem, Phaner et al. (25) dissolved a lipid extract mixture of various PL and TAG in a Folch solvent system modified with 20 mM ammonium formate to enable ESI-MS identification and quantification of each PL and TAG species. This technique decreased molecular species containing sodium ions in the crude lipid extract and lessened the possibility of isobaric mass overlap. Combining lipid profiling analysis using Direct flow Infusion ESI/MALDI along with FAMES will provide a thorough description of the effect of CLA-rich soy oil on the lipid composition in egg yolks.

Eggs enriched with 140 mg CLA per egg were produced at The University of Arkansas Poultry Farm using CLA-rich soy oil in a standard commercial hen diet and subsequently used in the following study. The goal of this study was to quantify differences in the fatty acid and lipid composition in CLA-rich eggs relative to control eggs. Fatty acid compositions were determined using FAMES by GC-FID. FAME studies by GC-FID were carried out directly from total lipid extracts and also from individual classes of lipids separated by TLC to estimate fatty acid composition differences. Lipid extracts were analyzed by direct MALDI-TOF-MS, Direct flow Infusion (DFI) ESI-MS, and TLC-UV-MALDI-MS to obtain fatty acid composition using the intact lipid form.

In summary the objectives of the investigation were to:

- 1) Determine fatty acid composition of total lipids in CLA-rich egg yolks relative to control eggs
- 2) Quantify the CLA content in TAG and PL fractions of CLA-rich egg yolks
- 3) Compare TAG and PL species in CLA-rich egg yolks and control yolks.

Materials and Methods

Materials

Control eggs were produced by adding 10% (wt) refined, bleached, deodorized (RBD) soy oil to a standard commercial diet for six commercial white leghorn hens, 25 weeks old. (Riceland Foods, Stuttgart, AR). CLA rich eggs were produced by adding 10% (wt) CLA-rich soy oil to a standard commercial feed for six additional commercial leghorns. The CLA oil contained 15% CLA. Diets were prepared by combining oil and feed in a Hobart stand mixer, and mixing at speed level 2 for 5 minutes (Hobart Legacy HL-200). CLA-rich eggs were collected after 12 days of treatment administration. CLA feeding was approved by the University's Institutional Animal Care and Use Committee.

All solvents used for sample preparation were analytical grade, and solvents used for TLC-UV-MALDI, Direct MALDI-MS, and Direct flow Infusion (DFI) ESI-MS were HPLC grade. The PL standards, including phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), lyso-phosphatidyl choline (LPC), and sphingomyelin (SM), were purchased from Cayman Chemical Co (Ann Arbor, MI). The fatty acid methyl ester (FAME) standards used were purchased from Supelco (Bellefonte, PA). Primuline dye was purchased from Sigma Aldrich (Cat. No. 206865-1G).

Methods

Fatty acid composition of total lipids

Yolk lipid extractions: Nine eggs were randomly collected from each of the CLA rich eggs and control eggs. Three egg from each egg type were pooled. This was performed three times to produce triplicate pooled samples. Eggs were carefully broken and a stainless steel yolk separator was used to separate the egg yolks for each replicate. Yolks were combined in 50 mL plastic centrifuge tubes and vortexed for 3 min to homogenize the pooled samples.

Lipids were extracted using a rapid hexane/isopropanol (IPA) solvent extraction protocol (26). Duplicate 5g samples from each yolk replicate were dissolved in ten times hexane/IPA (1:1, v/v), vortexed for 5 min,

filtered using Whatman #4 filter paper and the solvent evaporated using a roto-evaporator (Buchi Rotavapor R-210). The extract was weighed and yield recorded. These extracts were used to determine yolk total fatty acid composition, fatty acid composition of individual lipid classes, and lipid composition.

Fatty acid analysis from total lipids by GC-FID: FAMES were prepared from all yolk extractions using a rapid, micro FAMES preparation method (27). Samples of 0.1 g are weighed (Mettler Toledo Classic AB204-S) in 50-mL centrifuge tubes. A 1% heptadecanoic acid methyl ester (HME, C17:0; Sigma–Aldrich) solution in hexane was prepared. HME equivalent to 5% of the extract weight was added to each centrifuge tube as an internal standard. One milliliter of toluene and 4 mL of 0.5 M sodium methoxide in methanol were added to each tube. The tubes were heated to 50 °C in a water bath for 10 min then cooled at ambient temperature for 5 min. To inhibit sodium hydroxide formation, 0.2 mL of glacial acetic acid was added to each centrifuge tube. FAMES were efficiently extracted in to hexane by solvent extraction procedure. The tubes were vortexed for 2 min. and then placed on bench top for phase separation. The upper hexane layer was pipetted out and dried over anhydrous sodium sulfate for 15-20 s. Fatty acid profiles were obtained by measuring FAMES in duplicate by GC using an SP 2560 fused silica capillary column (100m x 0.25 mm i.d. x 0.2 µm film thickness; Supelco Inc., Bellefonte, PA) with a flame ionization detector (FID, model 3800, Varian, Walnut Creek, CA). Samples of 2.0 µL were injected by an auto sampler (Varian). The GC-FID settings were as follows: oven temperature = 250 °C, sensitivity = 12, He gas = 30 mL/min, H₂ = 31 mL/min, air = 296 mL/min, and oven program time = 111 min. Fatty acid concentrations were calculated by the following equation:

$$[\% \text{ fatty acid}] = ([\text{HME}] \times \text{sample peak area} \times \text{relative response factor}) / \text{HME peak area}$$

Statistical analysis were performed using JMP 10.1 software. Relative percentages of fatty acids were analyzed using a student t-test (α -level = 0.05).

Fatty acid composition of TAG and PL fractions

TLC-FAMES-GC-FID: Lipids were fractionated from yolk extraction samples using 20 x 20 cm TLC silica plates (T₂₅₄, Merck, Darmstadt, Germany). Samples of 0.3 g (\pm 0.005) were dissolved in 100 µL and applied to a TLC plate as 20 µL aliquots, carefully to prevent smearing. Four TLC plates were placed into a closed chamber with chloroform, ethanol, water and triethylamine (5:5:1:5) for approximately 2 hours to

perform chromatographic separation of all lipid classes. After separation the TLC plates were allowed to dry under the hood. Then one of the TLC plates were sprayed with 0.05% Primuline dye to visualize individual classes using Gel Logic GL2200 (Carestream Health, Inc. NY) (28). Once the locations of the TAG and PL on the TLC were determined, based on the primuline spray, TAG and PL fractions were scraped into separate 50 mL disposable centrifuge tubes and lipid extracts were converted to FAMES using the method described above (26). Autosampler injection volume was increased to 8.0 μ L to account for the lower sample concentration. Statistical analysis were performed using JMP 10.1 software. Relative percentages of fatty acids among egg yolk types were analyzed using a studentized t-test (α -level = 0.05).

Intact lipid composition

TLC-UV-MALDI analysis determination of TAG and PL species: Triplicate pooled samples from all three egg types plus a standard TAG and PL solution with a concentration of 50 mg/mL in CHCl_3 were applied to a TLC plate (TLC Silica Gel 60 5 x 7.5 cm, T₂₅₄, Merck, Darmstadt, Germany) as 1 μ L droplets to avoid smearing during the TLC loading. The mass of the dried sample loaded onto a TLC plate did not exceed 80 μ g. TLC plates were developed using chloroform, ethanol, water and triethylamine (5:5:1:5), for 45 min. After the separation was completed the TLC plate was allowed to dry under the hood. An airbrush sprayer attached to a high purity nitrogen tank was used to homogeneously coat the entire TLC plate with a 0.05% Primuline dye solution (28). The dye allowed visualization and quantification of lipid classes on TLC plate using UV excitation followed by monitoring 535 nm emission using Gel Logic GL2200 (Carestream Health, Inc. NY).

A separate TLC plate developed simultaneously with plates for UV analysis, as described above, was used for direct TLC-MALDI analysis. An airbrush sprayer attached to a nitrogen tank was used to homogeneously coat the entire TLC plate with a MALDI matrix aerosol (10 mL of 100 mg/mL DHB in acetonitrile/water, 4:1 v/v). After complete drying, the plate was fitted to a 5 x 7.5 MALDI TLC plate holder. TLC plate was imaged by Ultraflex II MALDI-TOF/TOF with 200 Hz smart beam laser in the reflector mode. The extraction voltage was set to 25 kV and matrix suppression was set to $m/z < 200$. Data were acquired using FlexControl (FC 3.0) and FlexImaging 2.0 software under Compass 1.2 settings. A pixel raster of 600 μ m x 600 μ m spots was set during the imaging run on the entire TLC lane

area. For each pixel 200 laser shots were accumulated. Certain colors were assigned based on the m/z to each lipid and the spatial distribution of lipids across the TLC plate was extracted from the MALDI-MS total ion count and displayed as a heat map. The data were processed using Bruker FlexImaging 2.0, Flex Analysis 2.4/3.0, and ClinProTools 2.2 software. Lipid species were identified by submitting both low and high resolution mono isotopic m/z peaks and the fatty acid composition information obtained from FAMES analysis to www.lipidmaps.org database.

Direct infusion ESI-MS and direct MALDI quantification of major TAG and PL species: A Bruker Esquire 2000 (Billerica, MA) LC ion trap mass spectrometer (Bruker, Daltonics Corp., Germany) was used to acquire all the mass spectra in this study. Triplicate pooled lipid extracts from each egg type were dissolved in chloroform to reach a final concentration of 50 mg/mL. Five μL of lipid extract was mixed with 200 μL of IPA/MeOH/ CHCl_3 (4:2:1, v/v/v) containing 20 mM ammonium formate (26). A 5 μL sample was directly infused into the electrospray ionization source for mass spectrometry analysis using a syringe. Mass Spectrometer was scanned from 400 to 1500 Dalton at a spectral averaging rate of 10 s^{-1} with low skimmer voltage optimized for ions at m/z 800. The mass spectrometer was operated in both positive and negative ion mode with a nebulizing gas pressure (N_2) of 12 Psi and a drying gas flow of 7 mL/min maintained at 150°C . The spectra data were analyzed by Bruker Daltonics Data Analysis 3.0 Software (Bruker Daltonik GmbH, Germany). Statistical analysis were performed using JMP 10.1 software. Relative percentages of lipid species among egg types were compared using a studentized t-test (α -level = 0.05).

Results and discussion

Fatty acid composition of total lipids

Fatty acid analysis from total lipids by GC-FID: Extractions resulted in a total lipid content of $34.7 \pm 0.05\%$ per volume of yolk with no significant difference in egg type. Table 1 shows the fatty acid composition of the control and CLA-rich egg yolk lipid extracts. Administration of the CLA-rich diet resulted in $2.02 \pm 0.08\%$ CLA of total fatty acids, equating to 140 mg CLA in the egg yolk. Myristic, palmitic and stearic acid concentrations, all saturated fatty acids (SFA), were significantly higher in CLA-rich eggs, relative to the control eggs ($p < 0.001$). Palmitoleic and oleic acid concentrations were significantly lowered in CLA-rich

eggs. These changes in SFA and monounsaturated fatty acid (MUFA) concentrations in response to dietary CLA have been reported multiple times (12-17). Linoleic and linolenic acid, both 18 carbon polyunsaturated fatty acids, were significantly higher in CLA-rich eggs. This increase corresponds to the increased linoleic and linolenic acid amounts in CLA-rich diet. Eicosenoic acid concentration was significantly higher in control yolks ($p < 0.001$).

Figure 1 shows fatty acid type composition for each treatment yolk. CLA-rich eggs contain 43.1% SFA, which is significantly higher than control eggs at 36.0% SFA ($p < 0.001$). PUFA concentrations in CLA-rich yolk was also significantly lower in comparison to both commercial egg types ($p < 0.001$). CLA-rich yolk MUFA concentration was 31.5%, which was significantly lower than both commercial egg types ($p < 0.001$). PUFA concentration was highest in CLA-rich yolks at 24.5%, while control eggs contained 14.0%. Long chain PUFA (LC-PUFA) concentrations were highest control yolks at 1.4% while CLA-rich yolks contained only 0.8%.

Fatty acid composition of TAG and PL fractions

TLC-FAMES-GC-FID: Table 2 shows the fatty acid composition of the TAG fraction of control yolk and CLA-rich yolk. Yolk lipids are generally comprised of 62% TAG and 33% PL and 5% cholesterol (29). The TAG fraction CLA content was 0.9% of total fatty acids. The relative proportions of CLA isomers in TAG reflected total lipid CLA isomers. CLA TAG had significantly more myristic, palmitic and stearic acid, relative to control TAG fractions ($p < 0.001$). Control yolk TAG had the highest concentration of C24:0 at 3%, which was significantly higher than CLA-rich yolk TAG.

Figure 2 presents TAG fatty acid concentration for control and CLA-rich yolk, grouped by degree of unsaturation. The relative proportions of fatty acid groups closely resemble what was seen in total lipid fatty acid composition. SFA concentration in CLA-rich yolk TAG was 46.9%, which was significantly higher than control commercial yolk at 37.7%. CLA-rich yolk TAG had significantly lower MUFA concentration at 35.4%, while control yolk TAG had 45%. Regular eggs yolks contained the highest levels of LC-PUFA in TAG at 3.6%, relative to CLA-rich yolk at 0.2%.

Table 3 shows the fatty acid composition of the PL fraction of control and CLA-rich yolks. Yolk lipids contained approximately 33% PL (29). The CLA contained in the PL fraction was 1.7% of total fatty acids.

CLA-rich yolk PL contained 20% stearic acid, which is significantly higher than control yolk PL at 16%. However, CLA-rich and control PL had no other significant differences in individual fatty acid concentrations.

Figure 3 depicts the PL fatty acid concentration for control and CLA-rich yolk, grouped by degree of unsaturation. CLA-rich yolk PL total SFA concentration was 45.8%, significantly higher than control yolk PL at 42.6%. Control yolk PL had significantly less SFA. The amount of MUFA in control regular yolk PL is surprisingly higher in control eggs at 59%.

Intact lipid composition

TLC-UV-MALDI analysis determination of TAG and PL species: Figure 4 depicts the separation and visualization of lipid classes by TLC-UV, including LPC, SM, PC, PI, PE and total TAG, which were validated by TLC-MALDI imaging mass spectrometry (image not shown), identifying two LPC species, one SM specie, 17 PC species, 19 TAG species and 9 PE species (Table 4) based on observed m/z values. PI species had relatively low ion intensities in positive ion mode as expected due to its ability to easily form a negative ion rather than positive ion under the MALDI positive reflector mode analysis and could not be identified. We did not see overall changes in lipids in CLA rich egg yolk compared to both commercial eggs.

Direct infusion ESI-MS and Direct MALDI quantification of major TAG and PL species: Both direct infusion ESI-MS and direct MALDI results lead to similar conclusions. Figure 5 shows representative average spectra for yolk lipid extracts. In the direct MALDI-TOF-MS spectra TAG were not detected due to PC suppression (22). When the lipid samples were fractionated by SPE before MALDI analysis, PL species intensities were much greater than TAG, although TAG are the more abundant in egg yolk lipids. ESI-MS resulted in a spectra of both TAG and PL species with an intensity ratio that was representative of the TAG and PL abundances. Direct flow Infusion ESI-MS in general had less peak intensity variation leading to lower standard deviation in estimating relative composition. Therefore, we used only the DFI ESI-MS data to calculate yolk lipid relative composition, shown in Table 4.

Lysophosphatidylcholine and sphingomyelin: LPC 16:0 and LPC 18:0 were significantly higher in CLA-rich yolks, relative to both controls ($p < 0.001$). Higher C16:0 and C18:0 levels were observed in CLA-rich yolk PL fractions, reported in Table 3. SM intensities were highest in control egg yolks ($p < 0.001$).

Phosphatidylcholine species: ESI-MS relatively quantified 15 PC species in the positive ion mode and 4 additional species in the negative ion mode. CLA-rich yolks had significantly higher PC C34:2, which contains linoleic and palmitic fatty acid chains. Palmitic and linoleic acid concentrations were higher in the CLA PL fraction, which explains this higher intensity. The PC C34:1 levels were not significantly different among CLA-rich and control yolks. This is despite the fact that oleic acid concentrations were significantly lower in CLA yolks. The PC C34:0 was highest in control yolk. This species was expected to be at higher concentrations in CLA due to the elevated SFA levels seen in the PL fatty acid profiles (Figure 3), but this was not the case. PC C36:4 contains two linoleic acid chains, and PC C36:3 and C36:2 both contain one. It was not surprising that these three species were at highest concentration in CLA-rich yolks, in response to the elevated total linoleic acid levels in the CLA-rich soy oil. The C36:2 concentration was even more elevated in CLA-rich yolks due to increased stearic, linoleic, and possibly CLA concentrations observed in PL fraction. PC C36:1 had two possible species identifications, one containing C16:0 and C20:1 chains, the other containing C18:0 and C18:1. Using the FAMES profiles for the PL fractions, it seems that the control yolk C36:1 contains only C18:0 and C18:1, while CLA-rich yolks have elevated C16:0 and C20:1 levels and could contain a mixture of both species. There were no significant differences between PC C38:6 and C38:5 among yolk types ($p = 0.3$). There were two PC species containing one docosahexaenoic acid chain. PC38:6 was identified in both positive and negative ion modes.

Triacylglycerols: CLA-rich yolk contained elevated levels of all TAG species containing only SFA (C48:0, C50:0, C52:0, and C54:0). This again coincides with the elevated saturated fat concentrations observed in CLA-rich yolks. A substantial amount of variation was found between species with 52 carbon chains. C52:5 and C52:4 were elevated in CLA-rich yolks. Using FAMES data with the www.lipidmaps.org mass spectra analysis tool, C52:5 and C52:4 were identified differently for control eggs than for CLA eggs. CLA yolks contain a C52:5 TAG species that has a linoleic, linolenic, and palmitic acid chain, while control

yolks have a species that has myristic, oleic, and arachidonic acid chains. It is likely that all three yolks have both isobaric mass species, and the database indicated the most probable specie based on the FAMES for each yolk type. The three fatty acids identified in C52:5 were all present at elevated levels in CLA yolks. The same rationale holds for the difference in C52:4 identifications for the three egg types. The C52:4 species contains two linoleic acid chains coupled with a palmitic chain. CLA-rich yolks contained the highest amount of this particular specie, and it is a likely candidate for CLA incorporation. This species was also significantly higher in CLA-rich yolks, but this specie more likely contains two linoleic acid chains and one oleic chain. The increased prevalence of this specie in CLA-rich yolk also indicates a potential candidate for CLA incorporation. Other TAG species containing one, two, or three oleic chains were significantly lower in CLA-rich yolk lipids (C54:4, C54:3, C54:2, C54:1) compared to control yolks.

Phosphatidylethanolamine species: All PE species were detected in the negative ion mode ESI-MS. CLA-rich yolks had significantly lower C36:1, C38:6, C38:5, and C38:4 compared to control commercial yolk PE. There were no significant difference in any of the PE species abundances between both commercial egg types.

In summary, The CLA rich eggs with 120 mg of CLA had significantly more saturated fatty acids and less monounsaturated fatty acids than did the control eggs. Comparable findings have been previously reported (12-18), but this study provided a comprehensive overview of intact lipid species and determined differences among CLA and commercially available eggs. The separated TAG fatty acid compositions of all three yolk types were similar to their respective total fatty acid composition. In CLA-rich yolks, 50% of the CLA was in the TAG and located predominately in species C52:5 and C52:4. Other TAG species containing linoleic acid were not at elevated levels. The PL fatty acid composition was not significantly different among egg types. All PC species containing one or two linoleic acid chains were most abundant in CLA-rich yolks. CLA-rich yolks contained more LPC than did the controls. Although CLA and control yolks did not show differences in total PE, CLA did lower the amount of certain PE species relative to the control eggs.

The TAG and total PL fractions contain similar amounts of CLA. A previous study using a commercially available CLA source reported preferential CLA incorporation in the TAG fraction versus PC and PE CLA content (19). Their method of lipid species fractionation did not enable them to ensure complete separation without possible contamination. TLC allowed complete separation and collection of species without any overlap. TAG fractions contained more saturated fatty acids and less polyunsaturated fatty acids than did the control egg yolks. CLA incorporation showed increased LPC concentration and modified the total PL composition. Although the yolk fatty acid composition is substantially different as a result of CLA incorporation, the relative composition of the lipid classes are maintained. Describing egg yolk lipids by both fatty acid analysis and molecular species analysis may help characterize the functional properties of different lipid species in egg nutrition studies.

Interest in egg yolk PC production for pharmaceutical applications has increased due to their high levels at 33% of total yolk lipids. This study illustrated that CLA enriched phospholipids and triacylglycerols are produced through the feed modification and may be isolated for further nutraceutical uses. This extensive yolk lipid profiling described how CLA incorporation affected other lipid species in the yolk. Providing the most comprehensive egg lipid profile to date may offer insight in further physiological and nutritional studies on CLA-rich and other designer eggs.

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Tables and Figures

Table 1. Fatty acid composition of yolk lipids from control egg and CLA-rich egg. Values are expressed as % of total fatty acids in crude lipid extracts. Values in the same row with the same letter are not significantly different (α -level = 0.05).

Fatty Acid	Control yolk fatty acids (% FA)	CLA-rich yolk fatty acids (% FA)
Myristic acid (C14:0)	0.33 ± 0.00b	0.39 ± 0.03a
Palmitic acid (C16:0)	24.46 ± 0.09b	26.21 ± 1.17a
Palmitoleic acid (C16:1)	1.33 ± 0.05a	0.84 ± 0.09b
Stearic acid (C18:0)	10.36 ± 0.05b	14.50 ± 1.16a
Oleic acid (C18:1)	32.49 ± 0.49b	27.32 ± 1.22b
Linoleic acid (C18:2)	24.27 ± 0.61b	26.24 ± 0.90a
Linolenic acid (C18:3)	0.72 ± 0.03b	0.93 ± 0.09a
CLA (C18:2 c9,t11)	0.00	0.13 ± 0.01a
CLA (C18:2 t9,c11 and c10,t12)	0.00	0.34 ± 0.01a
CLA (C18:2 t10,c12)	0.00	0.31 ± 0.02a
CLA (C18:3 other isomer)	0.00	0.08 ± 0.00a
CLA (C18:2 trans, trans)	0.00	1.17 ± 0.02a
Total CLA	0.00	2.02 ± 0.02a
Eicosenoic acid (C20:1)	1.69 ± 0.05a	1.57 ± 0.08b
Eicosatrienoic acid (C20:3)	0.18 ± 0.00a	0.17 ± 0.00a
Arachidonic acid (C20:4)	1.27 ± 0.05a	1.01 ± 0.03b
Docosahexaenoic acid (C22:6)	0.72 ± 0.01a	0.53 ± 0.10b

Figure 1. Total fatty acid composition of yolk lipids of yolk lipids from control eggs and CLA-rich egg. Fatty acids are grouped by degree of unsaturation: saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), long chain polyunsaturated fatty acids (LC-PUFA). Error bars are one standard error from the mean.

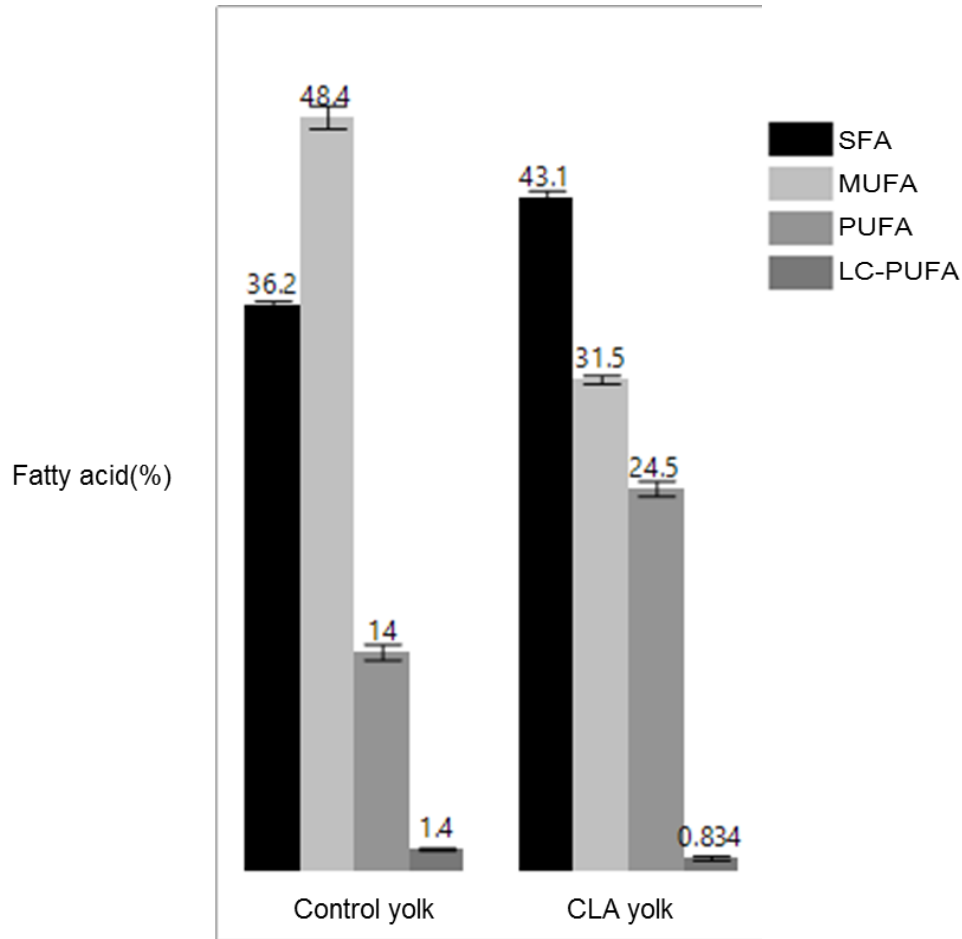


Table 2. Triacylglycerol (TAG) fatty acid composition of control yolk and CLA-rich yolk. Values are expressed as % of total fatty acids. Values in the same row with the same letter are not significantly different (α -level = 0.05).

Fatty Acid	Control yolk TAG fatty acids (%)	CLA-rich yolk TAG fatty acids (%)
Myristic Acid (C14:0)	0.21 ± 0.01b	0.49 ± 0.07a
Palmitic acid (C16:0)	23.15 ± 1.62b	32.07 ± 1.22a
Palmitoleic acid (C16:1)	1.61 ± 0.08b	1.1 ± 0.08c
Stearic acid (C18:0)	11.09 ± 1.04b	14.3 ± 0.49a
Oleic acid (C18:1)	43.23 ± 1.02a	34.2 ± 0.96b
Linoleic acid (C18:2)	13.42 ± 2.12b	16.06 ± 0.04b
Linolenic acid (C18:3)	0.33 ± 0.06b	0.46 ± 0.01b
CLA (C18:2 c9,t11)	0	0.18 ± 0.01a
CLA (C18:2 t9,c11 and c10,t12)	0	0.12 ± 0.02a
CLA (C18:2 t10,c12)	0	0.14 ± 0.02a
CLA (C18:2 t,t)	0	0.41 ± 0.07a
Total CLA	0	0.86 ± 0.11a
Eicosenoic acid (C20:1)	0.10 ± 0.04a	0.15 ± 0.01a
Eicosatrienoic acid (C20:3)	0.1 ± 0.03a	0.06 ± 0.01a
Arachidonic acid (C20:4)	0.42 ± 0.04a	0.16 ± 0.01b
Tetracosanoic acid (C24:0)	3.23 ± 0.18a	0.08 ± 0.004b
Docosahexaenoic acid (C22:6)	3.1 ± 0.79a	0.02 ± 0.002b

Figure 2. Triacylglycerol (TAG) fatty acid composition of yolk lipids from control yolk and CLA-rich yolk. Fatty acids are grouped by degree of unsaturation: saturated fatty acid (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), long chain polyunsaturated fatty acids (LC-PUFA). Error bars are one standard error from the mean.

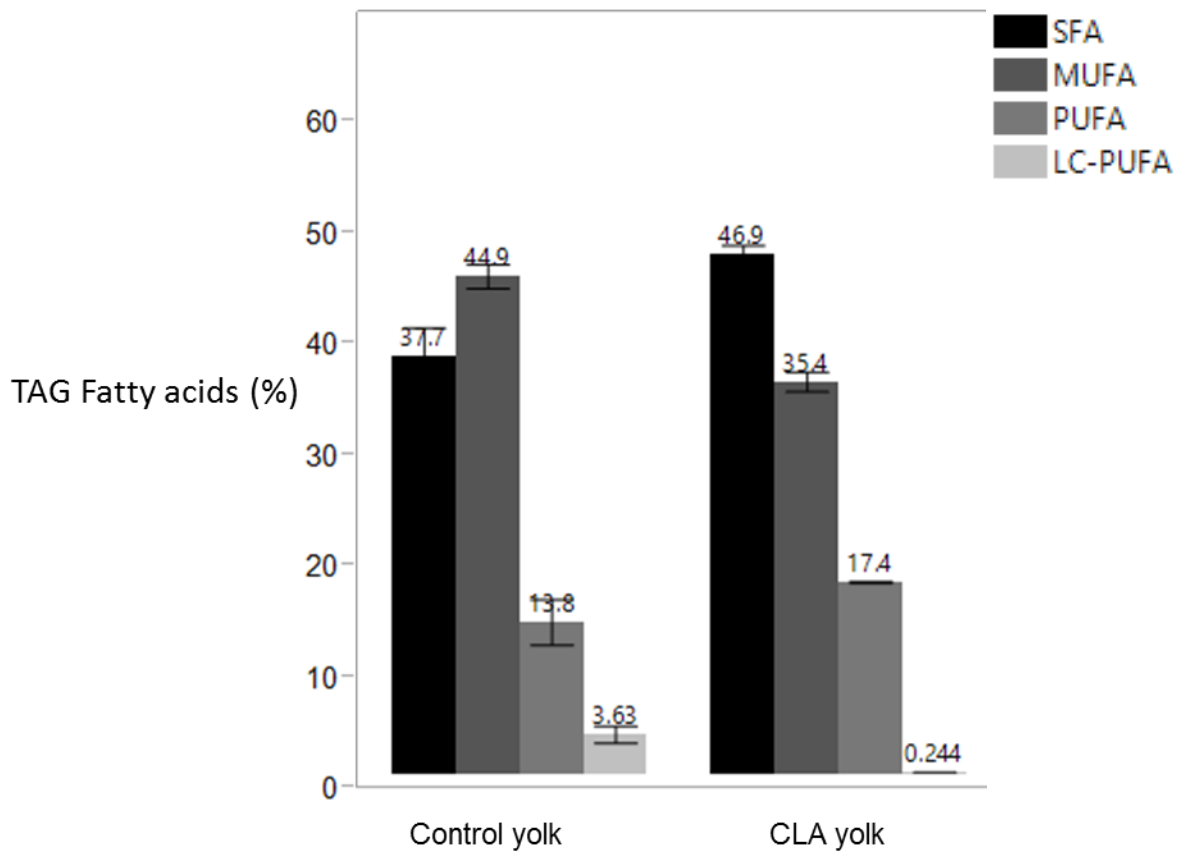


Table 3. Phospholipid (PL) fatty acid composition of control yolk and CLA-rich yolk. Values are expressed as % of total fatty acids. Values in the same row with the same letter are not significantly different (α -level = 0.05).

Fatty Acid	Control yolk PL fatty acids (%)	CLA-rich yolk PL fatty acids (%)
Myristic Acid (C14:0)	0.13 ± 0.01a	0.09 ± 0.02ab
Palmitic acid (C16:0)	15.29 ± 0.50b	23.83 ± 1.76a
Palmitoleic acid (C16:1)	1.86 ± 0.07a	0.35 ± 0.07c
Stearic acid (C18:0)	8.77 ± 0.10c	20.22 ± 0.41a
Oleic acid (C18:1)	56.67 ± 0.59a	25.61 ± 1.43b
Linoleic acid (C18:2)	15.25 ± 0.16b	18.82 ± 0.81a
Linolenic acid (C18:3)	0.36 ± 0.01a	0.33 ± 0.09a
CLA (C18:2 c9,t11)	0	0.34 ± 0.08a
CLA (C18:2 t9,c11 and c10,t12)	0	0.17 ± 0.06a
CLA (C18:2 t10,c12)	0	0.19 ± 0.03a
CLA (C18:2 t,t)	0	0.96 ± 0.01a
Total CLA	0	1.66 ± 0.06a
Eicosenoic acid (C20:1)	0.13 ± 0.01b	0.27 ± 0.03a
Eicosatrienoic acid (C20:3)	0.13 ± 0.01b	0.54 ± 0.13a
Arachidonic acid (C20:4)	0.9 ± 0.04b	4.94 ± 1.58a
Tetracosanoic acid (C24:0)	0.31 ± 0.02b	1.61 ± 0.52a
Docosahexaenoic acid (C22:6)	0.21 ± 0.01c	1.73 ± 0.59b

Figure 3. Phospholipid (PL) fatty acid composition of: Control commercial egg yolk, CLA-rich yolk, and omega-3 commercial yolk. Fatty acids are grouped by degree of unsaturation: saturated fatty acid (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), long chain polyunsaturated fatty acids (LC-PUFA). Error bars are one standard error from the mean.

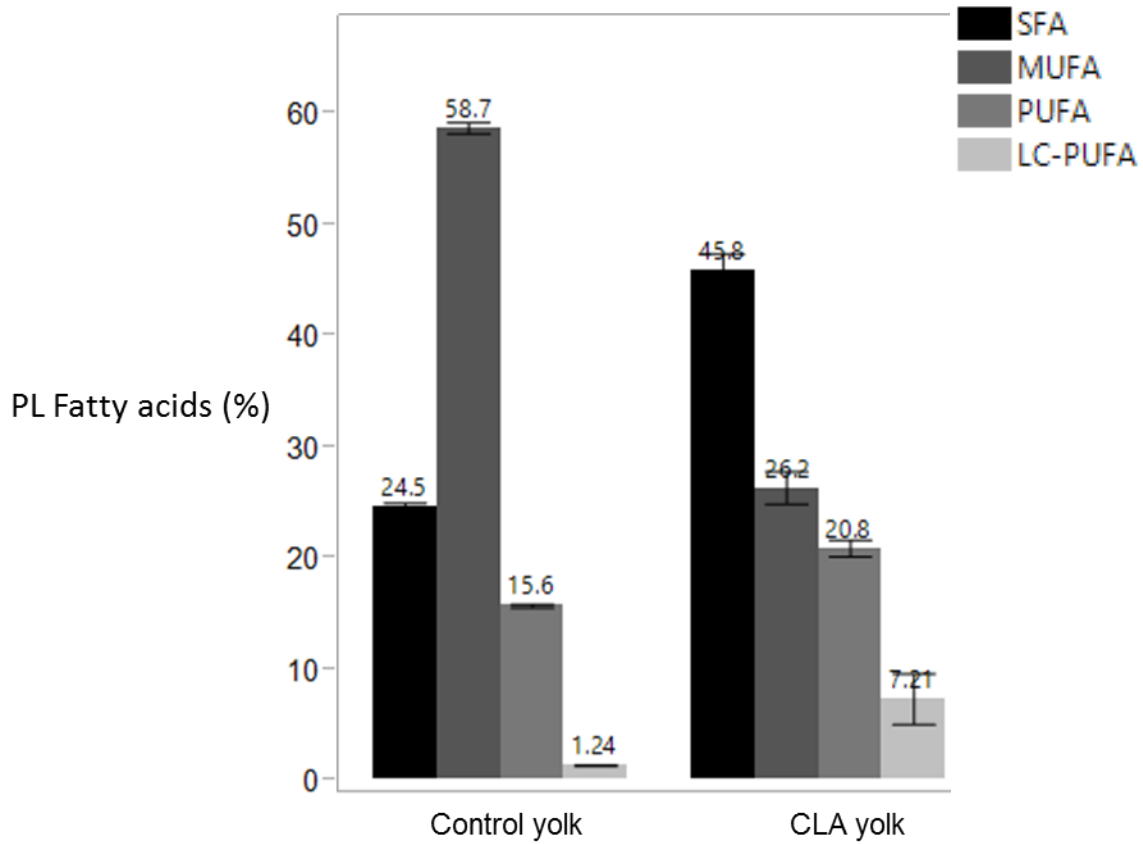


Figure 4. Lipid species separations from three yolk types: Control regular commercial yolk, CLA-rich yolk, and omega-3 commercial yolk. These images were developed from the primuline sprayed TLCs as described in the method section.

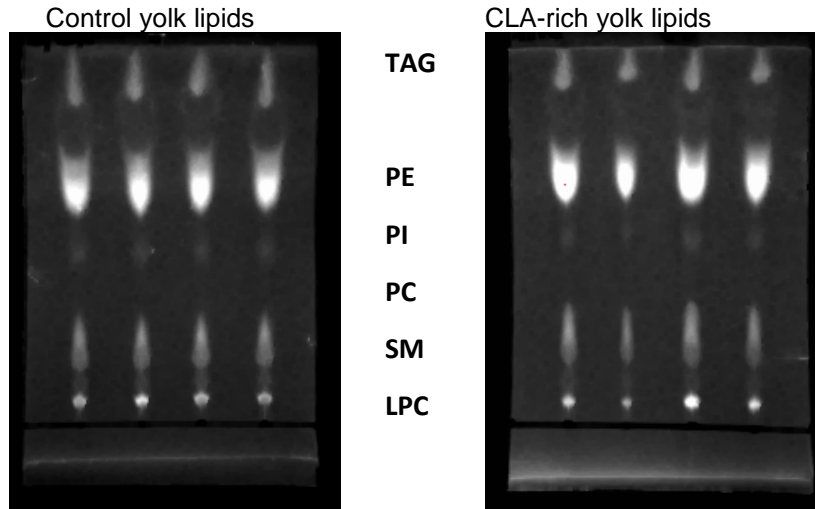


Table 4. Lipid species identified by TLC-MALDI and intensities quantified by ESI-MS, including lysophosphatidylcholine (LPC), sphingomyelin (SM), phosphatidylcholine (PC), triacylglycerols (TAG), and phosphatidylethanolamine (PE). Values in the same row with the same letter are not significantly different ($P = 0.05$). Lipid species with m/z (-) indicates species was detected in negative ion mode. Values in the same row with the same letter are not significantly different (α -level = 0.05).

LPC and SM Lipid			
Species	<i>m/z</i>	Control yolk	CLA yolk
LPC 16:0	496.3	0.48 ± 0.03b	0.95 ± 0.23a
LPC 18:0	524.3	0.29 ± 0.03b	0.84 ± 0.12a
SM 16:0	703.6	0.71 ± 0.04a	0.61 ± 0.06ab

Table 4. Lipid species identified by TLC-MALDI and intensities quantified by ESI-MS, including lyso-phosphatidylcholine (LPC), sphingomyelin (SM), phosphatidylcholine (PC), triacylglycerols (TAG), and phosphatidylethanolamine (PE). Values in the same row with the same letter are not significantly different ($P = 0.05$). Lipid species with m/z (-) indicates species was detected in negative ion mode. Values in the same row with the same letter are not significantly different (α -level = 0.05) (Cont.).

PC Lipid Species	<i>m/z</i>	Control yolk	CLA yolk
C34:2 [PC(16:0/18:2)]	758.57	5.98 ± 0.09a	7.99 ± 1.03a
C34:1 [PC16:0/18:1)]	760.58	13.97 ± 0.23a	12.06 ± 1.59a
C34:0 [PC(16:0/18:0)]	762.60	1.78 ± 0.06a	1.59 ± 0.04b
C36:4 [PC(18:2/18:2)]	782.57	1.71 ± 0.10a	1.84 ± 0.22a
C36:3 [PC(18:1/18:2)]	784.59	1.36 ± 0.07a	1.61 ± 0.23a
C36:2 [PC(18:0/18:2)]	786.60	3.61 ± 0.24a	5.15 ± 0.70b
C36:1 [PC(16:0/20:1) or (18:0/18:1)]	788.62	3.26 ± 0.31 ab	4.29 ± 0.34a
C38:7 [PC(22:6/16:1)]	802.54 (-)	12.76 ± 0.38a	13.62 ± 3.09a
C38:6 [PC(22:6/16:0)]	804.55 (-)	22.51 ± 0.74a	16.52 ± 3.66a
C38:6 [PC(22:6/16:0)]	806.57	0.73 ± 0.03a	1.09 ± 0.15a
C38:5 [PC(18:2/20:3)]	808.59	1.14 ± 0.26a	1.15 ± 0.25a
C38:4 [PC(18:0/20:4)]	810.60	2.02 ± 0.07ab	2.40 ± 0.29a
C38:3 [PC(18:0/18:0)]	812.62	0.56 ± 0.04a	0.64 ± 0.11a
C40:5 [PC(20:4/20:1)]	836.62	0.61 ± 0.16a	0.58 ± 0.01a
C40:7 [PC(22:6/18:1)]	830.57 (-)	5.62 ± 0.33a	6.35 ± 1.11a
C40:6 [PC(22:6/18:0)]	832.59 (-)	4.98 ± 0.58a	4.4 ± 0.71a
C38:1 [PC(20:1/18:0)]	838.63	0.11 ± 0.03b	0.22 ± 0.02a

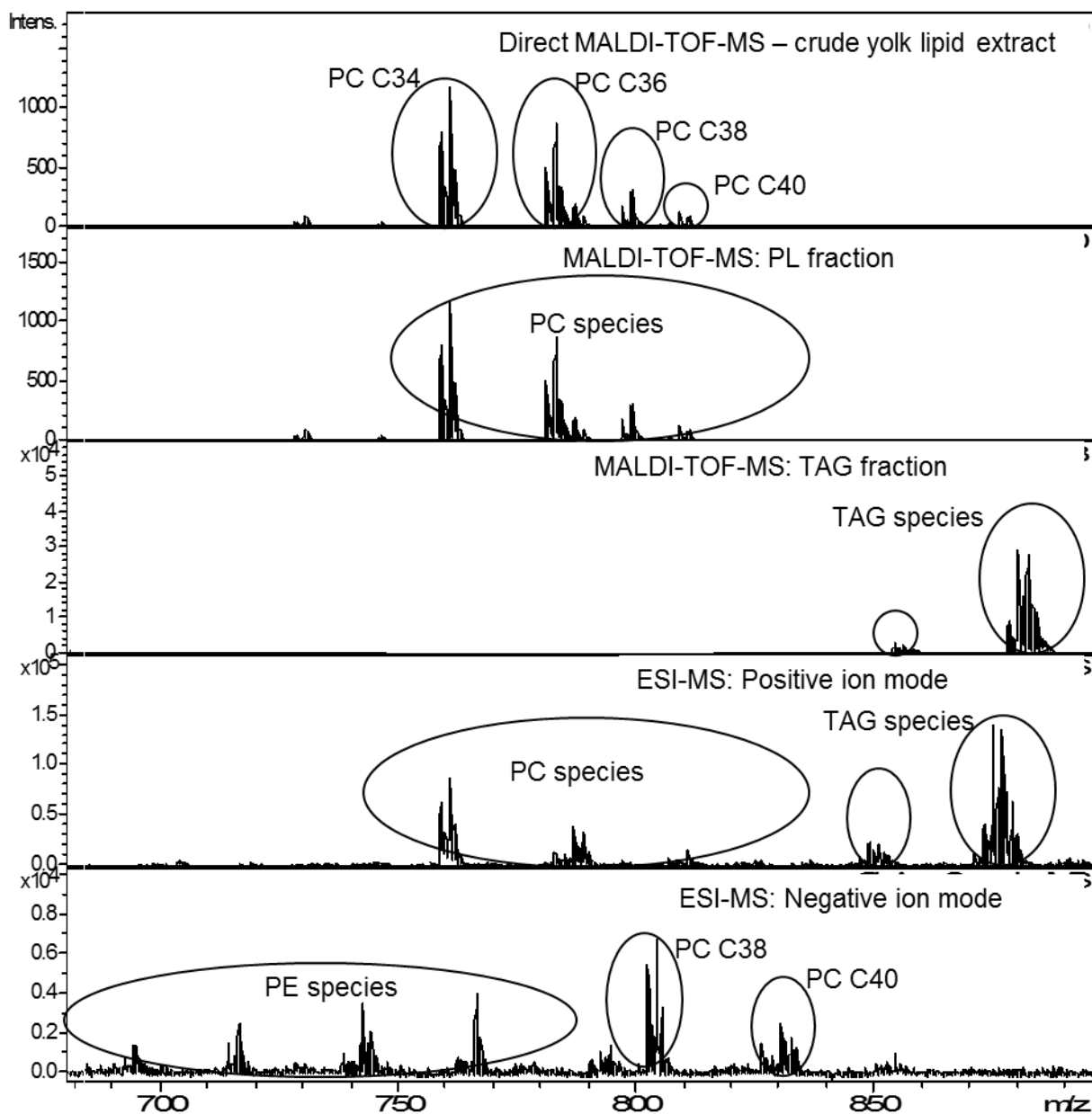
Table 4. Lipid species identified by TLC-MALDI and intensities quantified by ESI-MS, including lyso-phosphatidylcholine (LPC), sphingomyelin (SM), phosphatidylcholine (PC), triacylglycerols (TAG), and phosphatidylethanolamine (PE). Values in the same row with the same letter are not significantly different ($P = 0.05$). Lipid species with m/z (-) indicates species was detected in negative ion mode. Values in the same row with the same letter are not significantly different (α -level = 0.05) (Cont.).

TAG Lipid Species	m/z	Control yolk	CLA yolk
C48:0 (16:0/16:0/16:0)	824.77	0.32 ± 0.07b	0.55 ± 0.09a
C50:3 (14:0/18:1/18:2) or (16:0/18:3/16:0)	846.75	1.59 ± 0.12a	0.95 ± 0.05b
C50:2 (18:1/14:0/18:1)	848.77	3.42 ± 0.17a	2.73 ± 0.25a
C50:1 (16:0/16:0/18:1)	850.78	3.30 ± 0.07a	2.61 ± 0.39ab
C50:0 (16:0/16:0/18:0)	852.80	0.67 ± 0.08b	0.98 ± 0.09a
C52:5	870.75	0.31 ± 0.08b (14/18:1/20:4)	± 0.04a (18:2/16:0/18:3)
C52:4	872.77	2.63 ± 0.30b (16:0/18:3/18:1)	5.05 ± 0.35a (18:2/16:0/18:2)
C52:3 (16:1/18:1/18:1)	874.78	13.62 ± 0.70b	13.15 ± 1.54b
C52:2 (16:0/18:1/18:1)	876.80	17.44 ± 0.34a	12.52 ± 1.35b
C52:1 (18:0/18:1/16:0)	878.82	5.70 ± 0.40a	5.41 ± 0.89a
C52:0 (18:0/16:0/18:0)	880.83	0.96 ± 0.10ab	1.49 ± 0.29a
C54:5 (18:3/18:1/18:1)	898.78	0.30 ± 0.07b (18:3/18:1/18:1)	0.90 ± 0.14a (18:2/18:2/18:1)
C54:4 (18:1/18:2/18:1)	900.80	1.10 ± 0.14c	1.60 ± 0.04b
C54:3 (18:1/18:1/18:1)	902.82	2.48 ± 0.12b	2.26 ± 0.15b
C54:2 (18:1/18:1/18:0)	904.83	3.74 ± 0.25a	2.25 ± 0.31b
C54:1 (18:1/18:0/18:0)	906.85	2.15 ± 0.23a	1.36 ± 0.03b
C54:0 (18:0/18:0/18:0)	908.86	0.55 ± 0.07b	0.83 ± 0.04a

Table 4. Lipid species identified by TLC-MALDI and intensities quantified by ESI-MS, including lyso-phosphatidylcholine (LPC), sphingomyelin (SM), phosphatidylcholine (PC), triacylglycerols (TAG), and phosphatidylethanolamine (PE). Values in the same row with the same letter are not significantly different ($P = 0.05$). Lipid species with m/z (-) indicates species was detected in negative ion mode. Values in the same row with the same letter are not significantly different (α -level = 0.05) (Cont.).

PE Lipid Species	m/z	Control yolk	CLA yolk
C34:2 [PE(18:2/16:0)]	714.51 (-)	3.25 ± 0.40a	3.4 ± 0.61a
C34:1 [PE(18:1/16:0)]	716.52 (-)	7.72 ± 0.12a	5.12 ± 1.05a
C36:4 [PE(18:3/18:1)]	738.51 (-)	3.14 ± 0.05a	2.7 ± 0.41a
C36:3 [PE(18:2/18:1)]	740.53 (-)	1.94 ± 0.06a	1.35 ± 0.25a
C36:2 [PE(18:1/18:1)]	742.54 (-)	5.73 ± 0.39a	6.15 ± 1.87a
C36:1 [PE(18:0/18:1)]	744.55 (-)	7.16 ± 0.49a	4.8 ± 1.02b
C38:6 [PE(22:6/16:0)]	762.51 (-)	2.57 ± 0.48ab	1.56 ± 0.32b
C38:5 [PE(18:1/20:4)]	764.52 (-)	3.48 ± 0.05a	1.84 ± 0.31b
C38:4 [PE(18:1/20:3)]	766.54 (-)	12.35 ± 0.4a	8.03 ± 1.97b

Figure 5. CLA-rich egg yolk lipid extract spectra resulting from different MS analysis. In direct MALDI-TOF-MS the presence of PC suppressed TAG peaks. Fractionating the PL and TAG eliminated suppression. ESI-MS in positive ion mode allowed quantification of TAG and PC species, while negative ion mode allowed quantification of PE.



CHAPTER 4. Three hen strains fed photoisomerized *trans, trans* CLA-rich soy oil exhibit different yolk accumulation rates and source-specific isomer deposition

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Abbreviations

CLA	conjugated linoleic acid
<i>c,t</i>	<i>cis, trans</i>
DHA	docosahexaenoic acid (22:6n-3)
EPA	eicosapentaenoic acid (20:5n-3)
FA	fatty acid
FAME	fatty acid methyl ester
GC-FID	gas chromatography-flame ionization detector
HME	heptadecanoic acid methyl ester
MUFA	monounsaturated fatty acid
PUFA	polyunsaturated fatty acid
RBD	Refined, bleached, deodorized
SFA	saturated fatty acid
<i>t,c</i>	<i>trans, cis</i>
<i>t,t</i>	<i>trans, trans</i>

Abstract

Most CLA chicken feeding trials used *cis, trans (c,t)* and *trans, cis (t,c)* CLA isomers to produce CLA-rich eggs, while reports of *trans, trans (t,t)* CLA enrichment in egg yolks are limited. The CLA yolk fatty acid profile changes and the 10-12 days of feeding needed for maximum CLA are well documented, but there is no information describing CLA accumulation during initial feed administration. In addition, no information on CLA accumulation rates in different hen strains is available. The aim of this study was to determine a mathematical model that described yolk CLA accumulation and depletion in three hen strains by using *t,t* CLA-rich soybean oil produced by photoisomerization. Diets of 30-wk leghorns, broilers, and jungle fowl were supplemented with 15% CLA-rich soy oil for 16 days, and eggs were collected for 32 days. Yolk fatty acid profiles were measured by GC-FID. CLA accumulation and depletion was modeled by both quadratic and piecewise regression analysis. A strong quadratic model was proposed, but it was not as effective as piecewise regression in describing CLA accumulation and depletion. Broiler hen eggs contained the greatest concentration of CLA at 3.2 mmole /100 g egg yolk, then jungle fowl at 2.9 mmole CLA, and leghorns at 2.3 mmole CLA. The *t,t* CLA isomer levels remained at 55% of total yolk CLA during CLA feeding. However, *t-10,c-12 (t,c)* CLA concentration increased slightly during CLA accumulation and was significantly greater than *c-9,t-11* CLA. Jungle fowl had the smallest increase in yolk saturated fat with CLA yolk accumulation.

Introduction

Poultry feeding trials have used *cis*, *trans* (*c,t*) and *trans*, *cis* (*t,c*) CLA isomers to obtain CLA-rich eggs [1-7], resulting in up to 11% CLA in yolk total fatty acids when chickens were fed 5% CLA [1]. The *c-9*, *t-11* CLA isomer has been shown to be preferentially incorporated into yolks, even when the isomer concentration in the feed is equal to that of *t-10*, *c-12* CLA [1, 3, 5]. CLA concentration plateaus and stabilizes after 11 days of CLA administration, corresponding to 10-12 day yolk follicle maturation to full ovulation [1-5]. CLA egg enrichments have resulted in increased saturated fat and decreased monounsaturated fat levels in the yolks [1-5]. While changes in yolk fatty acids and the time it takes eggs to reach maximum CLA is well documented, there is no information describing CLA accumulation in those initial 10-12 days of feed administration.

Reports on different hen breeds ability to incorporate CLA in their yolks is limited. One study showed that while both white leghorns and brown dwarf layer hen breeds incorporated the same CLA amounts in yolk, white leghorn hens had higher amounts of yolk *c,t* isomers and lower amounts of *t,c* CLA in comparison to brown dwarf hens. In addition, CLA isomer concentrations did not reflect the diets' isomer composition [3, 5]. Broiler hens have been supplemented with CLA, which resulted in 6% CLA in breast muscle lipids and 10% CLA in leg muscle lipids, and abdominal fat deposition was significantly reduced [6]. However, the study did not determine yolk CLA concentration.

Recently, *trans*, *trans* (*t,t*) CLA isomers have shown superior anti-carcinogenic and anti-atherogenic effects in mouse and rat models [8-11]. In a comparative *in vitro* study, various CLA isomers were compared for the strongest anti-carcinogenic effect on colon cancer cells [9]. The strongest inhibition was demonstrated by *t-9,t-11* CLA, followed by *t-10,c-12* CLA, *c-9,c-11* CLA, and *c-9,t-11* CLA, respectively. Reports of *t,t* CLA superior anti-carcinogenic activity in MCF-7 human breast cancer cells and rat mammary tumorigenesis have also been published [8, 10]. *Trans*, *trans* CLA isomers have not been extensively studied due to lack of availability, despite the health benefits of *t,t* CLA that are now being realized [11]. Thus, in a separate study [12], it was determined that CLA enriched phospholipids and triacylglycerols were produced in egg yolks from chickens fed *t,t* CLA-rich soybean oil produced by photoisomerization. This CLA-rich soybean oil was produced by photoisomerization of soy oil linoleic

acid, of which 70% of the CLA isomers are in *t,t* form [13]. *Trans, trans* CLA-rich soybean oil was fed to obese rats and significantly decreased LDL, cholesterol, and liver lipid levels [14].

All previous CLA egg accumulation studies have been conducted with *ct, tc* CLA mixtures and, therefore, studies with *t,t* CLA are needed. This can be readily done with CLA-rich soy oil. Furthermore, yolk CLA and individual CLA isomer accumulation as a function of feeding duration, hen breed, or CLA depletion rates after dietary CLA removal has not been reported.

The aim of this study was to determine a statistical model that best described CLA egg yolk accumulation and depletion following feeding *t,t* CLA-rich soybean oil to commercial leghorns, commercial broilers, and jungle fowl. The specific objectives were to 1) Compare statistical models that describe CLA accumulation and depletion rates in CLA-rich egg yolks of broilers, leghorns, and jungle fowl 2) Compare *t,t* CLA yolk accumulation with that of other CLA isomers from CLA-rich soybean oil across the three hen varieties, and 3) Determine the changes in fatty acid composition in response to *t,t* CLA yolk accumulation and breed.

Materials and Methods

Materials

Refined, bleached and deodorized (RBD) soybean oil for CLA-rich soybean oil production was obtained from Riceland Foods (Stuttgart, AR, USA). Resublimed iodine crystals were used as a catalyst for CLA-rich soybean oil production (EM Science, Cherry Hill, N.J., USA). A standard commercial feed was obtained from Cobb Poultry Feed Mill (Cobb- Vantress Inc., Siloam Springs, AR, USA). This feeding trial was approved by the Arkansas Institutional Animal Care and Use Committee (Protocol # 13033).

Methods

Model Comparisons describing CLA accumulation and depletion

CLA-rich Soy Oil Production: A CLA-rich soybean oil containing 16% CLA was produced by the method of Jain et al. [13]. In summary, RBD soybean oil was combined with 5% *Magnesol* (commercial magnesium silicate, wt./wt.) and mixed using a stir-bar for 20 minutes (The Dallas Group of America, Inc.,

Whitehouse, NJ). In a previous study it was found that Magnesol adsorption removed large quantities of phospholipids and peroxides from soy oil and greatly increased CLA yields [15]. The solution was filtered using Whatman #4 filter paper with aid of a vacuum pump. The oil was then heated to 55°C and 0.25% of resublimed iodine was added [13]. The oil was then exposed to UV radiation for 12 hours [13]. Table 1 shows the fatty acid concentration of the original RBD soy oil and CLA-rich soy oil.

Dietary Treatments: The feed used for the study was a pelleted commercial corn and soybean meal-based finisher diet provided by Cobb-Vantress (Siloam Springs, AR). Feed was formulated without meat or animal by-products to meet or exceed minimum NRC (1994) standards for all ingredients. Nutrient levels of the unmodified feed are shown in Table 2. The control diet was prepared by adding 10 % (wt.) RBD soybean oil to the commercial feed. Ten percent CLA-rich soybean oil (% wt.) was added to the commercial feed for the CLA experimental diet. Diets were prepared using a Hobart stand mixer (Hobart Legacy HL 200) to adequately mix oil with feed. Fresh feed was produced every three days, and CLA-rich soybean oil was flushed with nitrogen and stored at 4°C to inhibit lipid oxidation.

Experimental Design Ten commercial white Cobb broilers, 10 Cobb white leghorns, and 10 full-colored jungle fowl, all 30-weeks-old, were used. Hens were randomly assigned to individual cages and separated by an empty cage to avoid stealing feed. Cages were fitted with individual feeding trays. Five hens from each breed were each fed 145 g / day of the CLA-rich diet, and the other five birds received 145 g / day of the control diet. Feed consumption was monitored throughout the study to ensure that all diets were completely consumed daily. Hens were fed the treatment diet for 16 days, and then returned to the control diet for an additional 16 days.

Eggs were collected from each hen variety and treatment group on days 1, 3, 5, 7, 8, 9, 10, 12, 14 and 16. Duplicate eggs from each hen variety were chosen at random and used to determine CLA accumulation. Similarly, duplicate eggs from each hen variety were collected on days 18, 19, 20, 21, and 23-26 were used to determine CLA depletion from duplicate yolks from each treatment group. All eggs were stored at 4°C for no more than 36 hours before lipid extraction and fatty acid analysis.

Total Lipid Extraction Total yolk lipids were extracted from duplicate eggs from each collection day and breed using a rapid hexane/isopropanol method [16,19]. Duplicate 4g yolk samples were dissolved in 40mL hexane/isopropanol (1:1, v/v), vortexed for 5 min, and filtered using Whatman #4 filter paper. The solvent was evaporated using a roto-evaporator (Buchi Rotavapor R-210) and extract was weighed.

Fatty Acid analysis Fatty acid methyl esters (FAMES) were prepared from all extractions using a rapid, micro FAMES preparation method [17]. Extraction samples of 0.1g were weighed (Mettler Toledo Classic AB204-S) in 50 mL centrifuge tubes. A 1% (wt.) heptadecanoic acid methyl ester solution (HME) in hexane was prepared and added at 5% of the extract weight as an internal standard. One mL of toluene and 4 mL of 0.5 M sodium methoxide in methanol were added to each tube [17]. The centrifuge tubes were heated to 50°C in a water bath for 10 min and then cooled at ambient temperature for 2 min. Glacial acetic acid, 0.2 mL, was added to each tube to inhibit sodium hydroxide formation. Five mL of distilled water was added, followed by 5 mL of hexane. The tubes were vortexed for 2 min and then the immiscible layers were allowed to separate. The upper layer was pipetted out and dried over anhydrous sodium sulfate for 15-20 s. Duplicate fatty acid profiles were obtained from each extract by measuring % FAMES by GC using an SP 2560 fused silica capillary column (100m x 0.25 mm i.d. x 2 µm film thickness; Supelco Inc. Bellefonte, PA) equipped with a flame ionization detector (FID model 3800, Varian, Walnut Creek, CA). Samples of 2.0 µL were injected by autosampler. The FID settings were as follows: oven temp = 250°C; sensitivity = 12, He gas = 30 mL/min, H₂ = 31 mL/min, air = 296 mL/min, initial column temp = 130°C, final column temp = 225°C, rate = 0.9°C/min, and program time = 111 min. Total fatty acid composition, CLA concentration and CLA isomer concentration were calculated by the following equation:

$$[\% \text{ fatty acid}] = ([\text{HME}] \times \text{sample peak area} \times \text{relative response factor}) / \text{HME peak area}$$

Fatty acid percent concentration were converted to mmoles of fatty acid/100 g yolk based on the molar mass of each fatty acid and lipid extract as constituting 32% of total yolk weight. The position of double bonds in CLA-rich soybean oil produced by photoisomerization [13] were previously determined by silver ion HPLC [18].

Statistical analysis The following statistical analyses were performed using JMP 11.0.1 software.

CLA concentration in yolk lipids during the steady-state period (day 12 to 16) were analyzed by 2-way analysis of variance (ANOVA). The LSMEANS, SEM and P values of the collection day, breed and day x breed, were determined to verify that CLA concentration stabilized after 12 d of CLA administration.

Quadratic model: Total yolk CLA accumulation and depletion by breed was first modeled using a 2nd degree polynomial fit, because the data points were in the shape of a parabola. An equivalence test was used ($\alpha = 0.05$) to compare the parameter estimates for the quadratic model from each hen variety.

Piecewise model: CLA accumulation and depletion were subsequently modeled as separate sub-functions in order to determine if CLA yolk accumulation rates were the same as depletion rates. In addition, accumulation and depletions rates were compared among hen varieties. Non-linear and linear 3-parameter models were separately fit to the accumulation and depletion segments of the data. The best fitting model was determined by comparing R² values, root mean square error (RMSE) values, and residual diagnostics were performed to ensure the final model was adequately representing the data. Predictions from the quadratic model and piecewise model were compared to the observed CLA concentrations. Rates of accumulation and depletion, and total yolk CLA concentration were analyzed by ANOVA in response to breed.

Comparison of trans, trans CLA yolk accumulation with other CLA isomers across breeds

The isomeric forms of CLA in CLA-rich soybean oil produced by photoisomerization [13] have been previously determined by silver ion HPLC [18]. Individual CLA isomer accumulations were modeled by linear regression, and analyzed by ANOVA as a response of breed and collection day. CLA isomer concentrations were compared using Tukey's Honestly Significant Difference Test at α -level of 0.05.

Yolk fatty acid compositional changes with respect to breed and collection date

Yolk total fatty acid composition after full CLA accumulation was analyzed by ANOVA in response to breed and blocked by collection date, and compared among varieties using Tukey's Honestly Significant Difference Test at α -level of 0.05.

Results

Model Comparisons describing CLA accumulation and depletion

Fatty acid analysis revealed that CLA was not detected in yolks until 6 days after CLA was added to the diets. On day 12 broilers' egg yolks reached a maximum of 0.031 mmol CLA per g of yolk, which was significantly greater ($P < 0.001$) than jungle fowl at 0.027 mmol CLA/g yolk, and leghorns at 0.023 mmol CLA/g yolk. CLA concentration in yolk lipids from day 12 to 16 did not increase (Table 3). On day 16 CLA supplementation was stopped, and CLA was no longer detected in yolks after day 23. CLA accumulation and depletion were subsequently modeled using both a quadratic model, and a piecewise model in order to determine which best described these rates.

Quadratic model: Figure 1 shows the total CLA accumulation and depletion in eggs fitted with a quadratic model. Equations 1, 2 and 3 are quadratic predictions of the CLA concentration in response to collection day for broilers, jungle fowl, and leghorns respectively.

$$\text{Broiler [CLA] (mmol/g yolk)} = - 0.01 + 0.006 \cdot \text{day} - 0.0002 \cdot \text{day}^2 \quad (1)$$

$$\text{Jungle fowl [CLA] (mmol/g yolk)} = - 0.007 + 0.005 \cdot \text{day} - 0.0002 \cdot \text{day}^2 \quad (2)$$

$$\text{Leghorn [CLA] (mmol/g yolk)} = - 0.006 + 0.04 \cdot \text{day} - 0.0001 \cdot \text{day}^2 \quad (3)$$

An equivalence test determined that there were significant differences in rates and total CLA accumulation and depletion among the three breeds ($P < 0.001$). The R^2 values for the broiler, jungle fowl and broiler models were 0.93, 0.93, and 0.94, respectively. The quadratic model accurately described the max CLA accumulation after 12 days of feed administration.

Piecewise model: The CLA yolk accumulation during days 1 through 12 is shown in Figure 2. Three different non-linear 3-parameter (3P) models were applied to yolk CLA accumulation data in JMP 11.0 software: cubic model, 3P logistic, and Gompertz 3P model. Using the root mean square error and R^2 values it was determined that CLA yolk accumulation was best modeled by the Gompertz 3P model (Eq. 4). The Gompertz 3P model had the smallest RMSE of all the three-parameter models evaluated at 0.23,

while the cubic and logistic models had larger RMSE values of 0.26 and 0.27, respectively. The Gompertz 3P model had an R^2 of 0.96, while the cubic and logistic models' R^2 were lower than 0.95.

The Gompertz 3P model is a time series model where growth is slowest at the start and end of a time period.

$$y(t) = ae^{be^{ct}} \quad (4)$$

Where a is the upper asymptote, b is the growth rate, c is the inflection point, and b and c are both negative numbers. Equations 5-7 are the predictive models for CLA accumulation in egg yolks of broilers, jungle fowl, and leghorns respectively.

$$\text{Broiler [CLA] (mmol/g yolk)} = 0.036 * e^{-(0.31 * e^{\wedge} - (5.98 * \text{day}))} \quad (5)$$

$$\text{Jungle fowl [CLA] (mmol/g yolk)} = 0.026 * e^{-(0.57 * e^{\wedge} - (4.50 * \text{day}))} \quad (6)$$

$$\text{Leghorn [CLA] (mmol/g yolk)} = 0.022 * e^{-0.48 * e^{\wedge} - (4.69 * \text{day})} \quad (7)$$

The R^2 values for these three prediction equations were all 0.96. The Gompertz 3P model adequately represents the lag time from day 1 to about day 3 of CLA accumulation, and CLA concentration stabilization after a growth period. These features of the actual data were not apparent in the initial quadratic model, and were not adequately represented by a cubic or logistic three-parameter model (models not shown).

Analysis of means of the three parameters provided in the Gompertz 3P model concluded that: 1) The asymptote estimate for broiler CLA accumulation model was significantly higher than leghorns and jungle fowl at the 0.05 α -level and 2) there was not a significant difference among CLA accumulation rates at the 0.05 α -level, and 3) The broiler's CLA inflection point was significantly higher than leghorns and jungle fowl at the 0.05 α -level.

Figure 3 depicts CLA depletion once hens were removed from CLA diets after 16 days of feeding. Depletion was best modeled by a cubic polynomial fit, indicated by the lowest RMSE of 0.16, compared to 0.25 and 0.29 for the Gompertz 3P and logistic 3P models, respectively (models not shown). Equations 8-10 are the cubic descriptive models for CLA depletion in egg yolks from broilers, jungle fowl, and leghorns respectively.

$$\text{Broiler [CLA] depletion (mmol/g yolk)} = 0.45 - 0.06 * \text{day} + 0.34 * \text{day}^2 - 0.006 * \text{day}^3 \quad (8)$$

$$\text{Jungle fowl [CLA] depletion (mmol/g yolk)} = 0.59 - 0.08 * \text{day} + 0.39 * \text{day}^2 - 0.006 * \text{day}^3 \quad (9)$$

$$\text{Leghorn [CLA] depletion (mmol/g yolk)} = 0.38 - 0.05 * \text{day} + 0.76 * \text{day}^2 - 0.004 * \text{day}^3 \quad (10)$$

The cubic fit also had R² values of 0.97 or greater, while the R² values for the other two models were less than 0.94. The cubic model seems most appropriate because it best described the steep CLA concentration drop determined in all three breeds after day 23, which corresponds to 7 days after CLA was removed from the diet. While the equivalence test determined differences in all parameter estimates among the three breeds, significant differences in the values were not determined due to so much more variability in CLA concentration among yolk lipids from the same chicken variety during CLA depletion.

Comparison of *trans, trans* CLA yolk accumulation with other CLA isomers across breeds

Figure 4 shows the accumulation rates of yolk CLA isomers. *Trans, trans* was the dominant isomer throughout CLA accumulation. Broilers contained significantly higher proportions of *t,t* CLA isomers when CLA was first detected on day 5 of the feeding trial at 56.3% of total CLA (P < 0.001). However, *t,t* CLA concentration in broilers decreased to 54.4% at peak CLA accumulation on day 12, which was not significantly different from *t,t* CLA concentration in leghorns or jungle fowl, which were also 54% of total CLA (p = 0.55). Jungle fowl and leghorns' initial *t,t* CLA concentration was 52% and 50% of total CLA isomers, respectively. Both breed's eggs experienced a slight increase in relative concentration until peak CLA accumulation on day 12. However, leghorn's egg yolks *t,t* CLA concentration was very variable throughout the CLA accumulation period. *Trans, trans* CLA isomers in the soybean oil constituted 72% of total CLA concentration.

Leghorn yolks had the greatest concentration of *c-9, t-11* CLA isomer at 14.7% of total CLA after 5 days of feeding, but its proportion of total CLA decreased significantly ($p < 0.001$) to 6.5% after 12 d. There were no significant differences in *c-9, t-11* CLA concentration on day 12 among the three hen strains, ranging from 6- 7% of total CLA ($p = 0.85$). The *c-9, t-11* CLA isomer was only 9% of total CLA in the CLA-rich soy oil.

The *t-9,c-11* and *c-10, t-12* isomer concentrations were found to be extremely variable throughout the yolk CLA accumulation period, but overall their relative concentration hovered around 16-17% of total CLA among all three breeds and all collection days. Broilers had significantly lower concentrations of *t-9,c-11* and *c-10, t-12* isomers ($p = 0.03$). These isomers were 12% of total CLA in the CLA-rich soybean oil.

The *t-10, c-12* CLA isomers' relative concentration significantly increased during the yolk CLA accumulation period in broiler and leghorns from initial concentrations of 14% to reach 18% of total CLA, while jungle fowl yolks concentration of this isomer did not waiver from 18% during CLA accumulation. The *t-10, c-12* CLA was only 7% of total CLA in the CLA-rich soy oil.

During days 12-16 there were no further observed changes in individual CLA isomer concentration. When hens were returned to a control diet on day 16, the concentration of individual CLA isomers during CLA clearance were so sporadic that no meaningful depletion rates for individual isomers could be drawn from the data.

Yolk fatty acid compositional changes with respect to breed and collection date

Table 4 shows the total fatty acid composition of RBD soy oil-rich egg yolks and CLA-rich egg yolks from leghorns, broilers, and jungle fowl after 12 d of feed administration. Soy oil –rich yolks from broilers had significantly lower 18:0 concentrations ($p < 0.05$) than leghorn yolks, but there were no other discernable differences in control yolk FA composition among hen strains.

Both the RBD and CLA-rich soy oil contain 16% SFA, but CLA-rich egg yolks had significantly greater saturated fat levels, relative to soy oil-rich yolks. Similarly, MUFA levels in both oils were the same at

26% of total FA, but CLA-rich yolks contained significantly lower MUFA levels, relative to control yolks. These fatty acid trends have been similarly reported in every CLA poultry feeding trial [1-6].

In addition, fatty acid concentrations in CLA-rich eggs differed among the three hen varieties (Table 4). Leghorn yolks contained significantly greater amounts of 18:0 relative to both broiler and jungle fowl yolks ($p < 0.001$). Leghorn yolks also had significantly lower 18:2n-6 levels than both jungle fowl and broilers ($p < 0.05$) as well as significantly lower 18:3n-3 concentrations than did broilers ($p < 0.05$). Although total MUFA levels were significantly lower in CLA-rich eggs versus control eggs, their concentrations did not differ among breed ($P = 0.47$), ranging from 30-31% of total fatty acids. Non-CLA polyunsaturated fatty acid concentrations were significantly lower in leghorn egg yolks compared to the other breeds ($P < 0.001$). Long chain polyunsaturated fatty acid levels were not different among hen breeds ($P = 0.18$).

Discussion

Model Comparisons describing CLA accumulation and depletion

The quadratic model and piecewise descriptive model both approximate CLA accumulation and depletion very well. However, quadratic models are intrinsically symmetrical, and will indicate that the maximum CLA concentration is reached only at the midpoint day of the feeding trial. In addition, this model assumes that the rates of CLA accumulation and depletion are equal, and did not allow a rate comparison. Furthermore, the quadratic model does not account for the empirical observation that CLA levels stabilize after 10-11 days of CLA administration [1-5]. It also does not accurately describe the depletion of CLA from the yolk after CLA was removed from the diets.

Alternatively, the piecewise model provided a more thorough description of accumulation and depletion rates because it incorporates the observed lag phase in CLA accumulation during days 1-4, and adequately describes CLA concentration stabilization at day 11. This model also accurately predicted the maximum yolk CLA concentrations from all hen strains. The piecewise model also describes total CLA depletion in yolks after CLA has been removed from the diet for 7 days.

The fact that both descriptive models were statistically robust shows the importance of observing both the mathematical and biological validity of a model. The piecewise model was not only statistically strong, but also accounted for features of egg laying biology such as 10-12 day cycle of yolk follicle maturation to full ovulation, which was not accounted for by quadratic modeling. Using a descriptive piecewise model allowed a determination and comparison of both accumulation and depletion rates among different hen varieties, which have not been previously reported. Since all studies that have modified the yolk fatty acid profile by supplementing different fats in the diet have reported a FA composition plateau after 10-12 days, it is likely that this piecewise model can be adapted to most fat-modified feeding trials.

Comparison of *trans*, *trans* CLA yolk accumulation with other CLA isomers across breeds

The *t,t* CLA isomer remained the predominant CLA isomer found in yolks of all three hen strains throughout the accumulation period. Higher concentrations of *t-10*, *c-12* CLA relative to *c-9*, *t-11* CLA were observed in the yolk, despite their opposite relative concentrations in the CLA-rich soy oil. This is different from a previous reports that supplemented equal amounts of *c-9*, *t-11* and *t-10*, *c-12* CLA, but observed a greater transfer efficiency of *c-9*, *t-11* to yolks, [2, 4, 22]. This CLA-rich soy oil seems to be absorbed differently, relative to CLA in free fatty acid form. CLA accumulation resulted in relative proportions of CLA isomers that were similar among the three breeds, with *t,t* CLA at the highest proportion, followed by *t-10*, *c-12*, *t-9*, *c-11/c-10*, *t-12*, and *c-9*, *t-11*.

The *t-10*, *c-12* isomer showed the most substantial increase in its relative proportion of total CLA during yolk accumulation. However, in previous studies, *t-10*, *c-12* and *c-9*, *t-11* CLA constituted the majority of the CLA isomers and were at similar concentrations in the diet, but *c-9*, *t-11* was always preferentially incorporated in the egg yolk at significantly higher concentrations, regardless of breed [1, 3, 5]. Other studies comparing different layer strains' ability to accumulate *c,t t,c* CLA in yolks reported preferential *c-9*, *t-11* CLA accumulation in white dwarf leghorns, while the *t-10*, *c-12* was preferentially incorporated in brown dwarf leghorns [3, 5] .

Yolk fatty acid compositional changes with respect to breed and collection date

While the transfer efficiency of the different CLA isomers were similar among hen strain, yolk total fatty acid composition was not consistent among strains. In chickens dietary lipids are transported the liver for further synthesis prior to subsequent tissue and yolk deposition. The stearoyl-CoA desaturase-1 is responsible for converting 16:0 to 16:1 and 18:0 to 18:1 [20]. Previous studies have deemed CLA as an inhibitor of this enzyme, which results in the subsequent increase of SFA in CLA-rich yolks [21, 22]. Broiler hen yolks had the lowest 18:0 levels when fed either the control or CLA-rich diet, relative to other hen strains. In addition, broiler had the greatest 18:2n-6 and 18:3n-3 yolk concentrations relative to the other bird strains. This suggests that CLA stearoyl-CoA desaturase-1 inhibition may not be as prevalent in broilers, relative to leghorns and jungle fowl.

Overall, broiler CLA-rich egg yolks seemed to exhibit the most desirable fatty acid alterations with the lowest increase in saturated fat concentration, as well as significantly higher MUFA and PUFA levels, and the greatest CLA concentrations in yolks. However, AA and DHA levels were lower in broiler eggs, relative to eggs of the other hen strains. It has been reported that DHA content is lowered in heart and brain tissue of hatched chicks whose mothers received CLA supplementation in free fatty acid form [21]. This study suggests that CLA effects on other tissue FA composition may very well be strain and CLA-source dependent.

It would be interesting to determine a hen strain whose metabolism is optimized for CLA-rich egg and meat production. CLA-rich soybean oil seems to be absorbed differently than other CLA sources and additional research on muscle and liver deposition is warranted. Furthermore, additional analysis on the specific health benefits of *t,t* CLA is needed before commercial production of this specific isomer is justified.

Descriptive modeling provides a unique insight as to how different CLA isomeric forms are deposited the yolk, as well as how that deposition changes as maximum yolk CLA concentration is reached. This statistical technique is applicable to most animal-product enrichment studies and may provide additional insight to the researchers and readers in this scientific field.

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Tables and Figures

Table 1. Fatty acid composition of refined, bleached, deodorized (RBD) soy oil, and resulting CLA-rich soy oil after 12 h photoisomerization, which resulted in 16% CLA.

Fatty acid	RBD Soy oil (g FA/ 100 g oil)	CLA-rich Soy oil (g FA/ 100 g oil)
Palmitic acid 16:0	12.2 ± 0.05	12.2 ± 0.02
Palmitoleic acid 16:1	0.09 ± 0.01	0.09 ± 0.01
Stearic acid 18:0	4.12 ± 0.04	4.24 ± 0.02
Oleic acid 18:1	25.0 ± 0.05	25.9 ± 0.06
Linoleic acid 18:2	55.0 ± 0.07	38.5 ± 0.05
Linolenic acid 18:3	3.54 ± 0.02	3.59 ± 0.02
<i>cis</i> -9, <i>trans</i> -11 CLA	ND ¹	1.44 ± 0.01
<i>Trans</i> -9, <i>cis</i> -11 & <i>cis</i> -10, <i>trans</i> -12 CLA	ND	1.89 ± 0.02
<i>trans</i> -10, <i>cis</i> -12 CLA	ND	1.12 ± 0.01
<i>trans</i> , <i>trans</i> CLA	ND	11.5 ± 0.02
Total CLA	ND	16.0 ± 0.03

Table 2. Percentage composition of the Cobb pelleted commercial feed that was used to prepare experimental diets. The feed used for the study was a pelleted commercial corn and soybean meal-based finisher diet provided by Cobb-Vantress (Siloam Springs, AR). Feed was formulated without meat or animal by-products to meet or exceed minimum NRC (1994) standards for all ingredients.

Metabolizable Energy (kcal/kg)	2761
Crude Protein (%)	15.43
Calcium (%)	1.45
Phosphorous (%)	0.43
Sodium (%)	0.15
Linoleic Acid (%)	0.15
Amino Acids	
Lysine (%)	0.61
Methionine (%)	0.27

Table 3. Maximum CLA concentration in egg yolks from broilers, jungle fowl and leghorn hens. Two-way ANOVA showed that CLA concentration did not increase after day 12 of treatment administration.

	CLA (mmole/g yolk)
	LSMEAN ± SEM
Broiler	0.031 ± 0.0002a
Jungle Fowl	0.028 ± 0.0002b
Leghorn	0.022 ± 0.0002c
	P-value
Breed	< 0.001
Day (12-16)	0.51
Breed x Day	0.91

Figure 1. Total CLA concentration in yolks from three hen strains during a 16 day administration of CLA-rich soybean oil feed, followed by a 12 day return to a control diet. Data was fitted with a quadratic model.

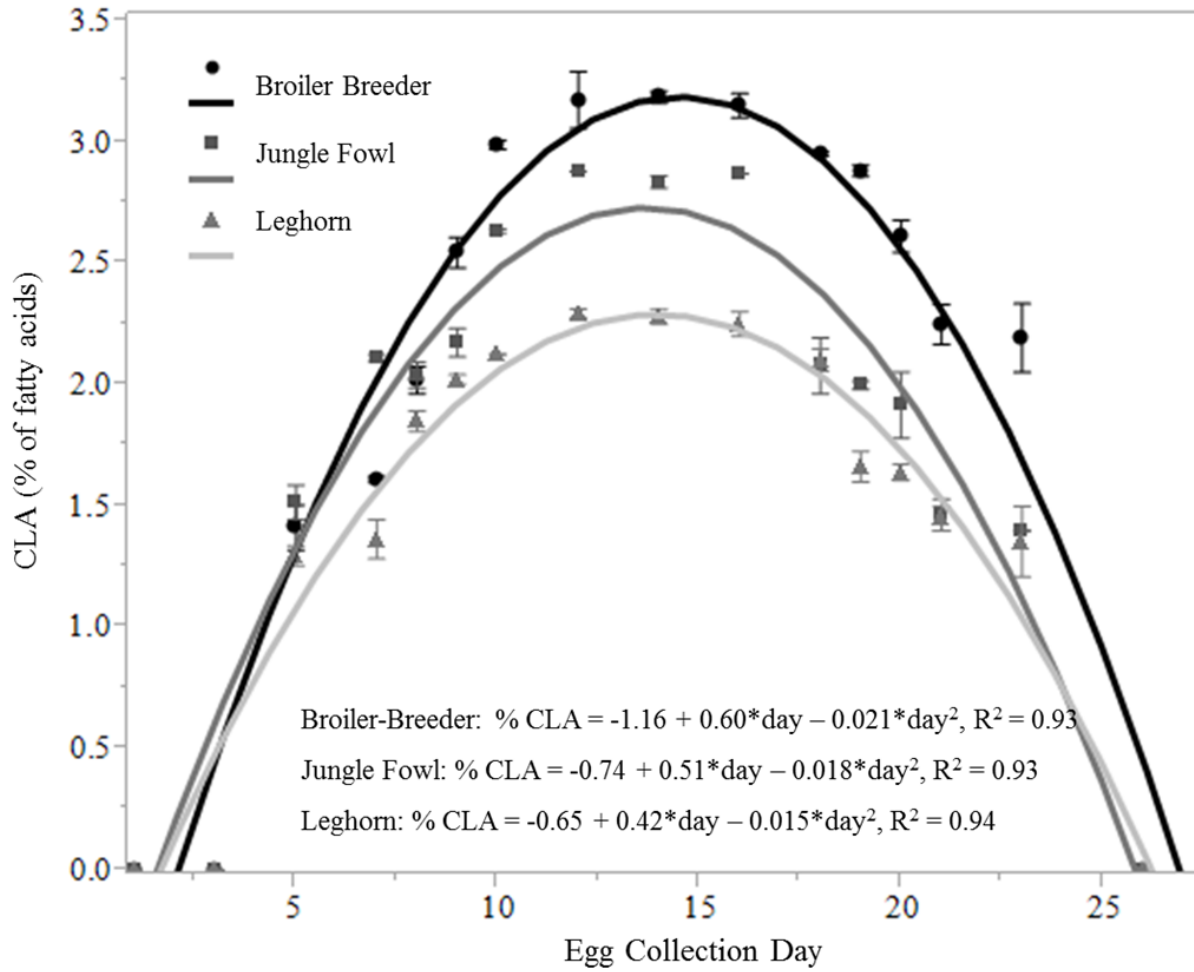


Figure 2. Total CLA accumulation in yolks from broilers, jungle fowl, and leghorns modeled by Gompertz 3-parameter model. Error bars are one standard error from the mean.

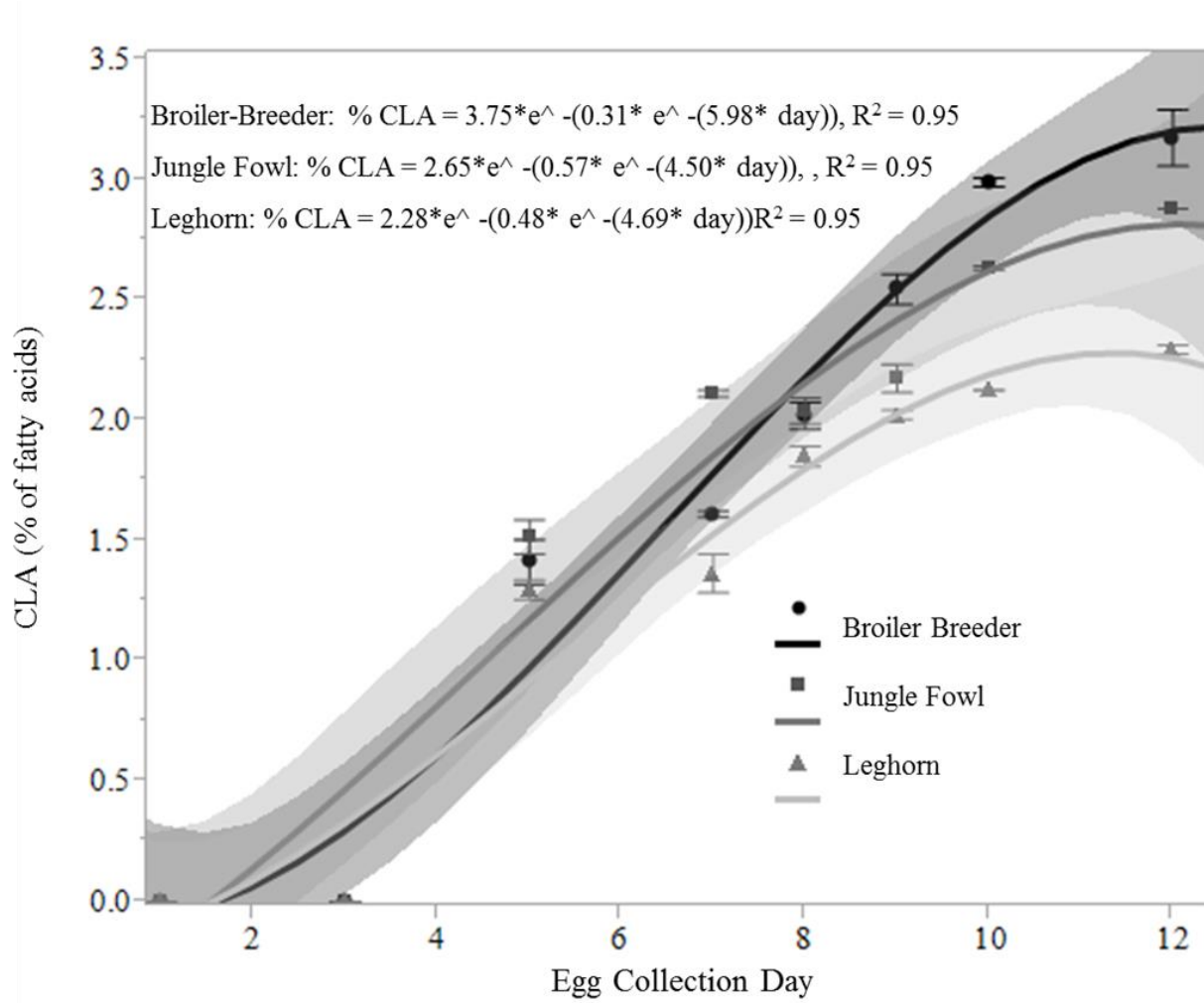


Figure 3. Total CLA depletion in yolks from broilers, jungle fowl, and leghorns modeled by a cubic equation. Error bars are one standard error from the mean.

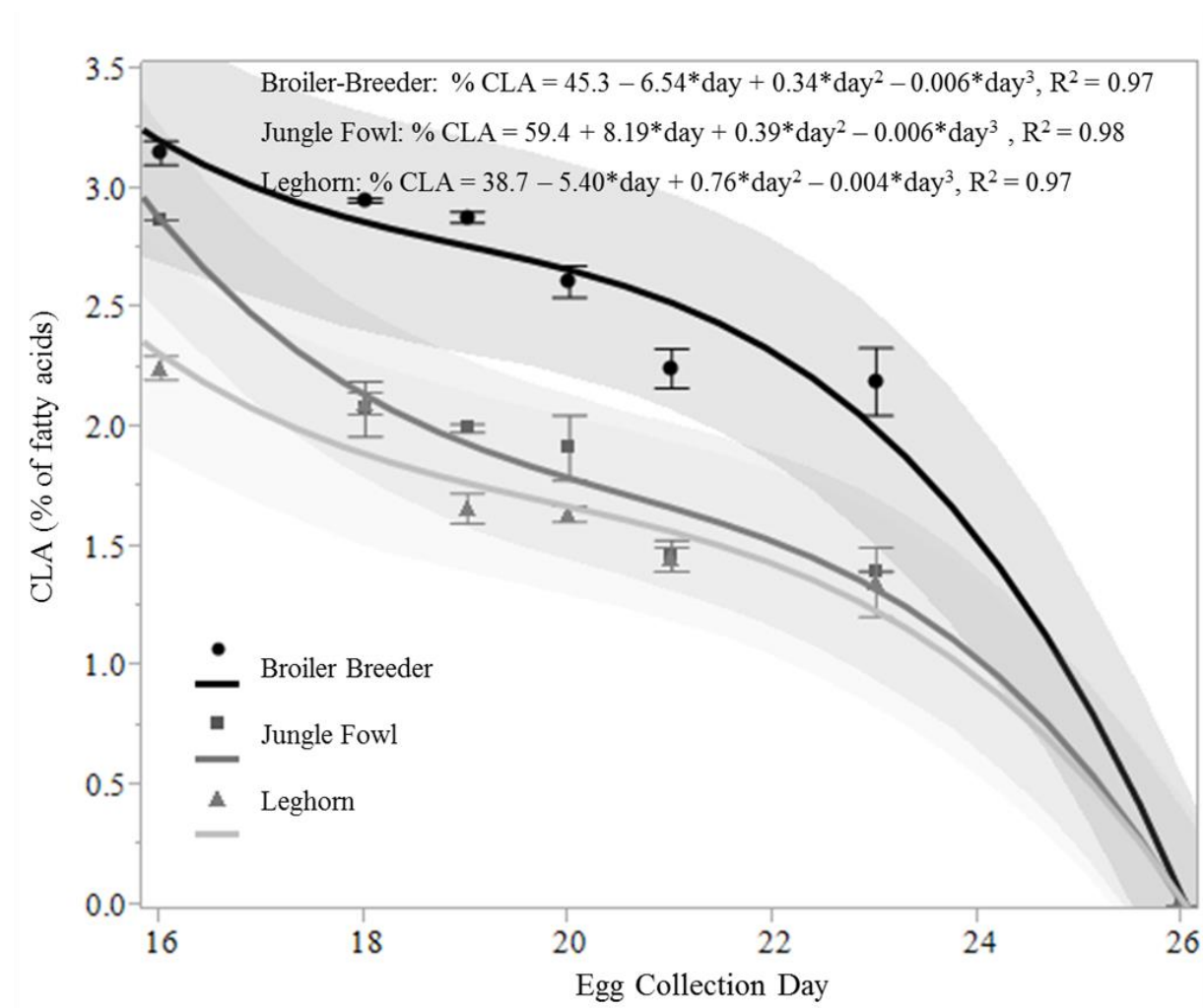


Figure 4. Egg yolk CLA isomer concentrations (mmol/g) during CLA accumulation in eggs of three hen strains consuming CLA-rich soybean oil diets. Accumulation rates were modeled by linear regression and comparisons among breed were determined by ANOVA.

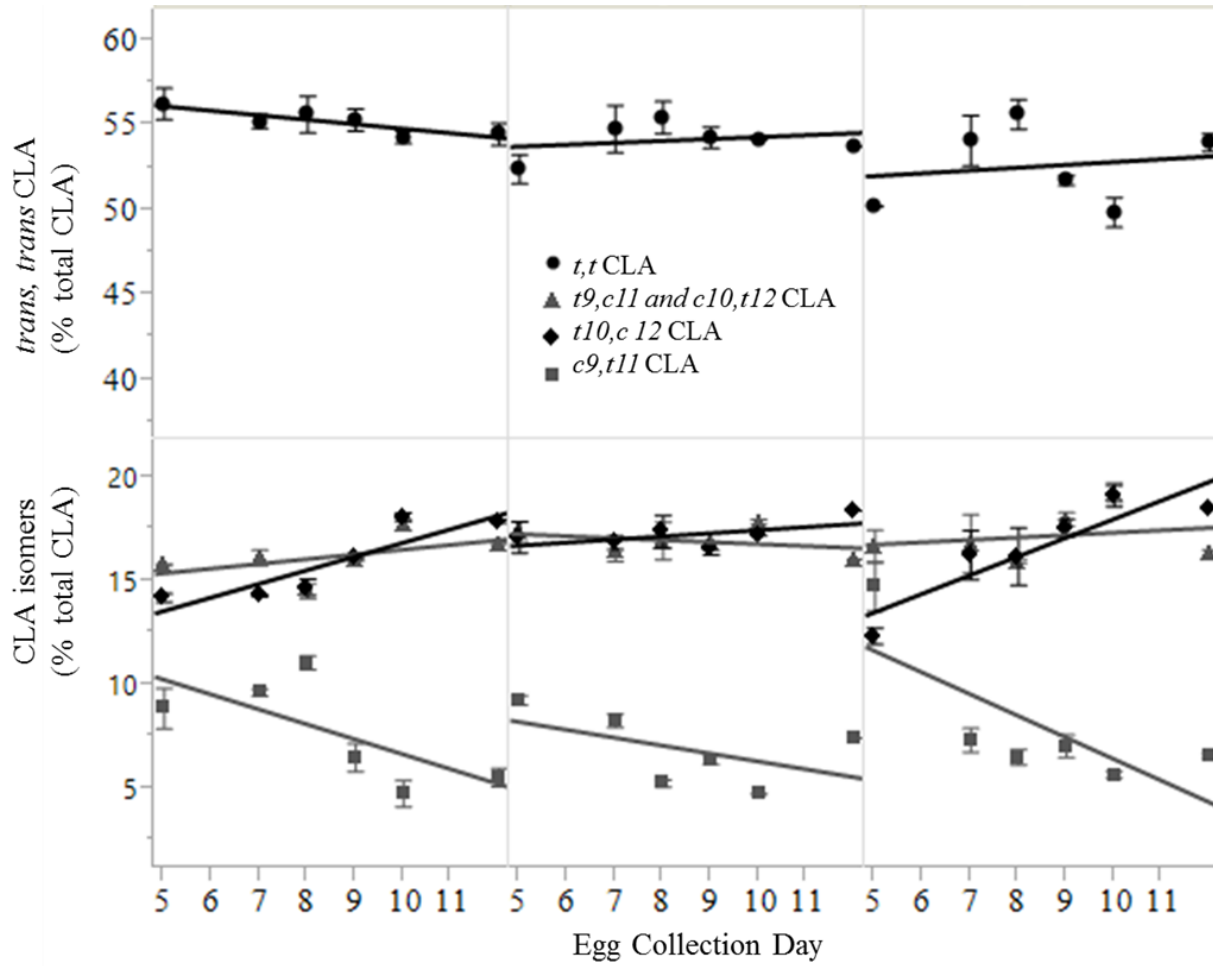


Table 4. Yolk total fatty acid composition from leghorns, broilers, and jungle fowl after 12 d of feeding. Significant differences (α -level 0.05) are shown by connecting letters within the same row.

A. RBD soy oil-rich egg yolk fatty acid composition from 3 hen strains

Fatty Acid (% mole of Total)	Leghorn	Broiler	Jungle Fowl
14:0	0.22 ± 0.02a	0.25 ± 0.03a	0.23 ± 0.02a
16:0	21.6 ± 0.27a	22.4 ± 0.69a	22.0 ± 0.30a
16:1	1.21 ± 0.02a	1.25 ± 0.03a	1.25 ± 0.02a
18:0	11.5 ± 0.27a	10.2 ± 0.30b	10.7 ± 0.36ab
18:1	36.5 ± 0.55a	38.0 ± 0.96a	36.4 ± 0.43a
18:2n-6	25.4 ± 0.81a	23.8 ± 0.62a	24.1 ± 0.29a
18:3n-3	0.78 ± 0.03a	0.85 ± 0.07a	0.80 ± 0.05a
20:4n-6	2.51 ± 0.29a	2.20 ± 0.08a	2.31 ± 0.35a
C22:6n-3	1.60 ± 0.13a	1.51 ± 0.07a	1.6 ± 0.10a
Total saturated fatty acids	33.3 ± 0.36a	32.8 ± 1.31a	32.9 ± 0.51a
Total monounsaturated fatty acids	37.7 ± 0.54a	39.2 ± 0.94a	37.7 ± 0.47a
Total polyunsaturated fatty acids	26.1 ± 0.21a	24.7 ± 0.62a	24.9 ± 0.47a
Total long chain polyunsaturated fatty acids	4.11 ± 0.45a	3.71 ± 0.21a	3.91 ± 0.44a

Table 4. Yolk total fatty acid composition from leghorns, broilers, and jungle fowl after 12 d of feeding. Significant differences (α -level 0.05) are shown by connecting letters within the same row (Cont.).

B. CLA-rich egg yolk fatty acid composition from 3 hen strains.

Fatty Acid (% mole of Total)	Leghorn	Broiler	Jungle Fowl
14:0	0.25 \pm 0.01c	0.40 \pm 0.02a	0.32 \pm 0.02b
16:0	28.6 \pm 0.24a	28.35 \pm 0.69a	29.0 \pm 0.34a
16:1	0.68 \pm 0.05a	0.84 \pm 0.06a	0.86 \pm 0.06a
18:0	17.4 \pm 0.29a	14.0 \pm 0.64b	14.7 \pm 0.36b
18:1	28.3 \pm 0.55a	29.03 \pm 0.96a	28.5 \pm 0.43a
18:2n-6	18.2 \pm 0.2b	20.8 \pm 0.62a	20.1 \pm 0.29a
18:3n-3	0.45 \pm 0.03b	0.76 \pm 0.07a	0.58 \pm 0.05ab
20:4n-6	2.57 \pm 0.29a	1.86 \pm 0.08a	2.33 \pm 0.35a
22:6n-3	0.82 \pm 0.13a	0.51 \pm 0.07a	0.6 \pm 0.10a
Total saturated fatty acids	48.81 \pm 0.36a	44.58 \pm 1.31b	46.37 \pm 0.51ab
Total monounsaturated fatty acids	29.97 \pm 0.58a	31.12 \pm 0.84a	30.55 \pm 0.47a
Total polyunsaturated fatty acids	20.58 \pm 0.27b	23.84 \pm 0.52a	22.88 \pm 0.47a
Total long chain polyunsaturated fatty acids	3.62 \pm 0.43a	2.59 \pm 0.11a	3.20 \pm 0.47a

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CHAPTER 5. Effect of trans, trans CLA egg enrichment from CLA-rich soy oil on yolk fatty acid composition, viscosity and physical properties

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Abstract

CLA egg accumulation studies using *cis, trans* (*c,t*) isomers have been effective, but reported adverse egg quality. *Trans, trans* (*t,t*) CLA isomers have shown superior nutritional effects in rodent studies, but reports of *t,t* CLA-rich yolks are limited. The objectives were to determine the effect of *t,t* CLA-rich soy oil in feed on egg yolk viscosity, and yolk quality during refrigerated storage. Yolk fatty acids, viscosity, weight, index, moisture, pH, and vitelline membrane strength (VMS) were determined at 0, 20, and 30 storage days. CLA had minimal effect on fatty acid profiles, relative to *c,t* reports. CLA-rich yolk viscosity was greater than controls, and CLA yolks maintained higher viscosities during storage. Yolk weight and index were not affected by *t,t* CLA-rich soy oil. Yolks with greatest CLA concentrations had the greatest VMS after 20 d storage, and yolks containing lower CLA levels maintained greater VMS throughout 30 d storage, relative to controls.

Introduction

Poultry feeding trials that reported conjugated linoleic acid (CLA) enriched eggs and subsequent egg quality changes have used predominantly *cis*, *trans* CLA isomer mixtures.¹⁻⁴ CLA supplementation of 4% and greater resulted in adverse egg quality effects such as egg, yolk and albumen weight reduction.⁵ Shell thickness and strength, yolk color and yolk index were negatively affected when CLA was the only lipid supplement.⁶ CLA egg enrichment induced an increase in yolk saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA) and decreased monounsaturated fatty acids (MUFA).^{1,2,4-6} Adverse effects on egg quality seemed to be reduced or prevented when CLA was co-supplemented with other oils, such as olive or soybean oil.⁶ Another prominent effect from CLA supplementation was elevated yolk pH when eggs were stored at refrigerated temperatures.⁷ This effect was not apparent when eggs were stored at room temperature.

CLA-rich egg quality changes were proposed to be due to increased yolk moisture content, and increased cation movement between yolk and albumen through the vitelline membrane.⁸ One study determined that dietary CLA caused calcium and zinc movement from yolk into albumen and subsequent sodium and magnesium migration from albumen to yolk.⁷ They hypothesized that modifications to the vitelline membrane allows minerals to move down the concentration gradient, and this alkaline migration may interact with hydrogen ions in the yolk, causing an increase in yolk pH. Vitelline membrane strength decreases significantly after only 2 weeks of refrigerated storage.²⁸ However, no studies have determined changes in vitelline membrane strength in response to CLA enrichment, or correlated such quality changes with yolk fatty acid composition, moisture content, pH alterations, or refrigerated storage durations. Furthermore, no studies have determined the effect of CLA enrichment on egg yolk viscosity. Usually egg yolk viscosity decreases during refrigerated storage conditions, so egg based food products are ideally prepared with freshly-laid eggs to obtain ideal texture and quality.

In the *trans*, *trans* (*t,t*) CLA isomer fatty acid chain substituents on each side of the double bonds are oriented in opposing directions, resulting in a straight fatty acid chain. There have been few reports on *t,t* CLA, but the importance and need for more research on this isomer has been recently recognized.⁹ Recent nutritional studies have demonstrated that *t,t* CLA isomers have superior anti-carcinogenic and anti-atherogenic effects in mouse and rat models in comparison to the more often studied *cis*,

trans mixtures.¹⁰⁻¹² A pilot scale photoisomerization process of linoleic acid in soybean oil was developed that produced up to 20 % total CLA.¹³ Approximately 75 % of total CLA were *t,t* isomers, while the remaining were in the *cis, trans* and *trans, cis* form.^{13,14} A subsequent animal nutrition study showed that *t,t* CLA-rich soy oil beneficially reduced fatty liver and lowered serum cholesterol in obese Zucker rats.¹⁵ This *t,t* CLA-rich soybean oil was subsequently used to produce CLA-rich eggs with predominantly *t,t* CLA in yolks.¹⁶ This study determined that both CLA-enriched phospholipid and triacylglycerols were present in the yolks. In addition, CLA-rich yolks had significantly higher concentration of completely saturated triacylglycerol species. CLA-rich eggs containing predominantly *t,t* isomers may exhibit quality and textural changes that have not been reported with feeding trials using *cis, trans* CLA mixtures. Total yolk lipid composition of CLA-rich eggs from mass spectrometry analysis¹⁶ in addition to fatty acid analysis may provide insight into reasons possible physical and viscosity changes observed in *t,t* CLA-rich eggs.

Previous egg enrichment studies either report the modified fatty acid composition relative to control eggs, or determine changes in egg quality. Correlations between egg quality alterations and fatty acid composition are rarely reported. The aim of this experiment was to investigate the effects of *t,t* CLA enrichment on egg quality during a typical shelf-life of 30 days refrigerated storage. In addition, correlations between CLA-egg yolk fatty acid composition and any resulting quality or viscosity changes were also explored. The objectives were to determine the effect of *t,t* CLA-rich soy oil in feed on egg yolk viscosity, and yolk quality during refrigerated storage. Yolk quality parameters that were investigated included yolk weight, yolk index, moisture content, pH, and vitelline membrane strength.

Experimental Section

Materials

All solvents used for sample preparation were analytical grade. The fatty acid methyl ester (FAME) internal standard and long chain polyunsaturated FAME standards used were purchased from Supelco (Bellefonte, PA).

Methods

CLA-Rich Soy Oil Production

A 18% CLA-rich soybean oil used in the feeding trial was produced by linoleic acid photoisomerization using a pilot scale unit and method of Jain & Proctor.¹³ The refined, bleached and deodorized (RBD) soybean oil was provided by Riceland Foods (Stuttgart, AR).

Feeding and Egg Production

Feed Preparation: CLA oil produced by photoisomerization (18% CLA) was combined with RBD soybean oil to produce treatment oils containing 15%, 10%, 5%, 2.5% and 0% CLA. The fatty acid composition for the soybean oil and CLA oil are provided in Table 1. The feed used for the study was a pelleted commercial corn and soybean meal-based finisher diet provided by Cobb-Vantress (Siloam Springs, AR). Feed was formulated without meat or animal by-products to meet or exceed minimum NRC (1994) standards for all ingredients. Treatment diets were produced by adding 10% (wt.) soy oil or CLA oil to the standard commercial feed and combined using a Hobart stand mixer.

Diet administration: Animal procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee (Protocol # 13033). One hundred single-comb white leghorn chicks were reared under standard commercial conditions. At 25 weeks, sixty hens were randomly chosen and assigned to single bird cages in blocks of 5 birds each, separated by 2 empty cages. All hens received the same standard commercial finisher diet until the feeding trial began at 33 weeks. Each soybean oil diet (15%, 10%, 5%, 2.5% and 0% CLA) was randomly assigned to 2 blocks of 5 birds each, resulting in 10 birds receiving each treatment diet. The remaining two hen blocks continued to receive standard commercial feed without additional soybean oil and served as a standard control group.

Egg Collection: After 12 days of treatment feed administration, eggs were collected daily, counted, and labelled with cage number and date for 20 days. Eggs were stored in a walk in cooler at 4°C. Eggs used for analysis were refrigerated for either 0, 5, 10, 20 or 30 days prior to analysis.

Fatty Acid Analysis

Yolk lipid extractions: Total yolk lipids were extracted from quadruplicate eggs from each treatment and storage duration [N=120] using a rapid hexane/isopropanol method.¹⁷ A 0.5 g yolk sample was collected

in a 7 mL glass vial and dissolved in 5 mL hexane/isopropanol (1:1, v/v), vortexed for 5 min, and allowed to settle. The liquid containing the lipid extract was decanted into a 50 mL plastic centrifuge tube and used for subsequent FAME preparation.

Fatty acid Analysis as FAMES: Fatty acid methyl esters (FAMES) were prepared from all extractions using a rapid, micro FAMES preparation method modified for the volume of lipid extract solution.¹⁸ Decanted extraction samples in 50 mL centrifuge tubes were combined with 0.5 mL of a prepared 1% (wt.) heptadecanoic acid (C17:0) methyl ester solution in hexane. Then 1 mL of toluene and 4 mL of 0.5 M sodium methoxide in methanol were added to each tube. The centrifuge tubes were placed in a 50°C water bath for 10 min with constant agitation and then removed and cooled at ambient temperature for 2 min. Glacial acetic acid, 0.2 mL, was added to each tube to inhibit sodium hydroxide formation. Five mL of distilled water was added, followed by 2 mL of hexane. The tubes were vortexed for 2 min and then the immiscible layers were allowed to separate. The upper layer was pipetted out and dried over anhydrous sodium sulfate for 15-20 s.

Duplicate fatty acid profiles were obtained for each lipid extraction by GC with a SP 2560 fused silica capillary column (100m x 0.25 mm i.d. x 2 µm film thickness; Supelco Inc. Bellefonte, PA), and a flame ionization detector (FID model 3800, Varian, Walnut Creek, CA). Samples of 3.0 µL were injected by autosampler. The FID settings were as follows: oven temp = 250°C; sensitivity = 12, He gas = 30 mL/min, H₂ = 31 mL/min, air = 296 mL/min, and over program time = 111 min. Total fatty acid composition, CLA concentration and CLA isomer concentration were calculated by the following equation:

$$[\% \text{ fatty acid}] = ([\text{HME}] \times \text{sample peak area} \times \text{relative response factor}) / \text{HME peak area}.$$

The position of double bonds in CLA-rich soybean oil produced by photoisomerization¹³ were previously determined by silver ion HPLC.¹⁴ Fatty acid percent concentration were converted to mmoles of fatty acid/100 g yolk based on the molar mass of each fatty acid and lipid extract as constituting 32% of total yolk weight.

Statistical analysis: Data were analyzed by ANOVA in response to dietary CLA concentration. Tukey's test was used to compare treatment means, which were deemed significant a $p \leq 0.05$. P-values comparing individual treatments were determined using orthogonal contrast analysis.

Viscosity analysis

Triplicate yolks from all six treatment groups (standard control, soy control, 15%, 10%, 5%, and 2.5% CLA) were analyzed in duplicate as either freshly laid yolks (0 day), or after 20 or 30 day refrigerated storage [N = 54]. In addition, triplicate control, soy control, and CLA-rich eggs containing the highest dietary CLA concentration were analyzed in duplicate after 5 and 10 days of refrigerated storage [N = 18]. An AR-2000 Rheometer was used for viscosity analysis.¹⁹ Each yolk sample was vortexed for 20 s and 2 aliquots of yolk were dispensed from a 1 mL auto pipette on the lower rheometer plate. A 40 mm diameter sand-blasted geometry was applied to the sample surface at a constant shear rate of 100 s⁻¹. The initial plate temperature was stabilized at 4°C for 2 min and then heated at a constant rate of 10°C/min until a final temperature of 60°C was reached. Sheer stress (Pa) measurements were recorded 100x per minute. Viscosity was calculated as shear stress divided by the constant sheer rate.

Statistical analysis: Yolk viscosity data has been determined to fit the Power Law in the temperature range of 4°- 60°C, and can be adequately modeled by a non-linear exponential 3-parameter model that describes the asymptote, viscosity at initial temperature, and decay rate (Equation 1).²⁰ Parameter estimates were compared by analysis of means at an alpha level of 0.05 in response to storage duration and CLA concentration.

(Eq 1)
$$\text{Viscosity} = A + B \cdot e^{(C \cdot \text{Temp})}$$

Where A = asymptote, B = scale, and C = decay rate

Correlations between viscosity at 4°C and fatty acid compositions were determined for 0, 20 and 30 day eggs. Correlation values 0.30 and above indicate a weak linear relationship, values 0.50 and above indicate a moderate linear relationship, and values above 0.70 indicate a strong linear relationship. If the value is negative the paired items are inversely correlated.

Egg Quality Analysis

Egg yolk physical properties: Duplicate eggs from duplicate treatment blocks were analyzed fresh (0 day), or after 20 or 30 days of refrigerated storage. Whole egg weights were measured and recorded, followed by yolk weights, which were weighed after being gently rolled on a Kim-wipe to remove any

adhering albumen and chalazae. Yolk index (YI), defined as the ratio of yolk height to diameter, was measured using a caliper and recorded.

Vitelline membrane strength (VMS): The force required to puncture through the yolk vitelline membrane was determined using TA-XT2i Texture analyzer equipped with a 5 kg load cell and a 1 mm probe.²⁷ The probe was calibrated at 18mm above the platform. The test speed was set at 3.2 mm/s and distance target mode was 13 mm. The peak force (g) was recorded as VMS for quadruplicate eggs from each treatment group on each refrigerated storage duration.

Yolk pH and moisture content: Yolk pH was measured in triplicate on day 0, 20 and 30 d eggs. Yolk moisture content was determined gravimetrically in duplicate eggs during the 30 d refrigerated. Yolks were dried in an oven at 100°C for 20 h.²¹

Statistical analysis: Data were analyzed according to a split-plot design, with dietary CLA concentration as the whole plot and duration of egg storage as the sub-plots, to determine main effects of dietary CLA and duration of storage, and their interactions. Tukey's test was used to compare treatment and main effect means, as appropriate. When dietary CLA by refrigeration duration interactions were observed, comparisons among treatments were made. Otherwise, comparisons were made among main means.

Results and Discussion

Fatty acid analysis

Total yolk CLA, CLA isomers and PUFA concentrations are presented in Table 2. Egg yolk total CLA increased linearly as dietary CLA levels increased ($\text{mmole CLA/g yolk} = 0.05 + 0.18 * \text{g CLA/kg feed}$, $R^2 = 0.8$). *Trans, trans* CLA was the most abundant isomer in all CLA-rich yolks, and increased linearly in response to total CLA in the diet ($\text{mmole } t,t \text{ CLA/g yolk} = 0.02 + 0.1 * \text{g CLA/kg feed}$, $R^2 = 0.77$). Yolks with the lowest CLA concentration had 42% of CLA in *t,t* form, while yolks with the greatest CLA levels had 53% in *t,t* form. The *c-9, t-11* CLA concentration was similar in all CLA yolks, but its proportion of total CLA decreased from 28% to only 5% in response to higher CLA levels in the diet. The proportion of *t-10, c-12* to total CLA was similar in all CLA yolks at about 15%, and its concentration increased linearly with CLA in the diet ($\text{mmole } t-10, c-12 \text{ CLA/g yolk} = -0.02 + 0.04 * \text{g CLA/kg feed}$, $R^2 = 0.80$). All other CLA isomers maintained similar proportions of total CLA, and increased linearly in response to dietary

concentration. However, previous studies reported that the *cis*-9, *trans*-11 CLA isomer was preferentially incorporated in CLA yolks, whereas the *t*-10, *c*-12 incorporation was less efficient.² In contrast, when *t,t* CLA-rich soybean oil was used in the feed, *t,t* CLA was preferentially incorporated, followed by *t*-10, *c*-12. Linoleic and linolenic acid concentrations were similar in all eggs produced from soy oil-rich diets, and were significantly greater than the standard control yolks ($p < 0.001$). While standard control egg yolks had the greatest arachidonic acid (AA) concentration at 0.17 mmole/100 g yolk, AA levels in the other treatment yolks had such a large variation that no significant differences could be determined relative to control yolks. Eicosapentanoic acid (EPA) concentration was greatest in yolks from the soy control diet, but its concentration decreased significantly with increasing CLA concentration ($p < 0.001$). However, yolks containing 0.5 mmole of CLA per 100 g yolk contained a similar amount of EPA when compared with standard control eggs. Docosahexanoic acid (DHA) concentration was also greatest in soy control yolks and decreased with increasing CLA concentration ($p \leq 0.003$). However, DHA levels in egg yolks with the greatest CLA concentration were similar to DHA levels in standard control egg yolks ($p = 0.60$). Previous studies have reported significantly lower long chain PUFA levels in CLA-rich eggs and have deemed this effect undesirable.^{1,2,8} Ahn *et al.* reported a decrease in AA concentration from 3.7% to 2.7% of total FA when hens were fed 2.5% CLA, while Chamruspollert *et al.* reported a more dramatic drop in AA levels from 3.9% to 2.6% of total FA from hens fed 2.5% CLA.^{1,8} However, this consequence of CLA enrichment seemed to be lessened using the *t,t* CLA-rich soybean oil, as standard control yolks contained 1.7% AA of total FA while CLA-rich yolks produced from 1.5% CLA in the diet lowered AA concentration to 1.4% of total FA (0.13 mmole CLA/100 g yolk). Likewise, Szymczyk and Pisulewski reported a DHA decrease from 1.4% to 0.2% of total FA with 2% CLA present in the diet. However, DHA levels in CLA-rich yolks and control yolks were not significantly different in this study. The reduction of these fatty acids and subsequent increase in stearic acid has been attributed to stearyl-coenzyme A desaturase inhibition in the liver.^{22,23} This lesser decrease in DHA may be preferable, when compared with eggs produced from feeding *cis,trans* mixtures.

Table 3 presents yolk saturated and monounsaturated fatty acid levels. Egg yolk total saturated fatty acid (SFA) content significantly increased as yolk total CLA concentration increased ($p < 0.001$), while soy control egg yolks had significantly lower saturated fat levels in comparison to standard control yolks ($p =$

0.02). A previous CLA feeding trials using *cis,trans* isomer mixtures observed a 34% increase in SFA levels with only 0.5% CLA in the diet.⁷ However, CLA-rich soybean oil raised SFA levels by only 28% with 1.5% CLA in the diet, relative to soy control yolks.

Table 3 also shows that egg yolk monounsaturated fatty acid (MUFA) concentration decreased significantly in yolks produced from all soy oil diets. The MUFA decrease became more substantial as yolk CLA concentration increased. Previous studies reported a 32% drop in MUFA concentrations.⁷ This study determined only 25% decrease in MUFA concentration in yolks containing the greatest CLA concentrations, relative to the control yolks. The overall trends in fatty acid composition modifications are similar to previous reports with a significant increase in saturated fatty acids and subsequent decrease in monounsaturated fatty acids.^{1,2,4,5} However, *t,t* CLA-rich soybean oil reduced these changes relative to previous studies.

Viscosity analysis

Fresh egg yolks: Table 4 shows the initial viscosity, asymptote (lowest part of curve), and average decay rate. Supplemental Figure 1 shows the differences in viscosity among yolks on days 0, 20, and 30. On day 0 yolks containing 2.7 mmoles of CLA had significantly greater initial viscosity than all other yolk types ($p < 0.05$). However, initial yolk viscosity was not consecutively lowered with respect to decreasing yolk CLA concentration. For example, egg yolks containing 0.9 mmoles CLA displayed a significantly lower initial viscosity compared to all other yolks. Analysis of means revealed that fresh egg yolks from soybean oil-enriched diets displayed viscosities that leveled off at similar values (asymptote) while standard control yolk leveled off at a significantly lower viscosity. The viscosity decay rates were faster in response to increasing yolk CLA concentration, while control and soy control yolks displayed similar decay rates.

Correlations between initial viscosity and fatty acid composition in day 0 yolks revealed that viscosity was moderately correlated with total SFA ($R = 0.43$), CLA concentration ($R = 0.54$), and moderately inversely correlated with EPA concentration ($R = -0.57$). The fatty acid composition shows that EPA concentration in 0.9 and 2.0 mmole CLA yolks are similar, and drops significantly in 2.7 mmole CLA yolks. It is not completely apparent why 0.9 mmole CLA yolks displayed such a low initial viscosity compared to all other

yolks, but it may be due to a synergistic effect of EPA and CLA levels, along with a SFA level that is not significantly different from control yolks.

Egg yolks refrigerated for 5 days: After 5 days of refrigeration CLA yolks has a significantly greater initial viscosity of 3 Pa-s than control yolks with an initial viscosity of 1.9 Pa-s, while soy control yolks had a significantly lower initial viscosity at 1.6 Pa-s than control yolks. The viscosity decay rate was significantly faster in CLA yolks, followed by soy control, and control yolks. Control yolk viscosity had a greater asymptote than CLA or soy control yolks, which had similar asymptotes.

Egg yolks refrigerated for 10 days: CLA yolks continued to have significantly greater initial viscosities in comparison to both the control and soy control yolks. However, both CLA and soy control yolks stored for 10 days had initial viscosities that were actually greater than their 5 day old counterparts. Although asymptote estimates provided by modelling indicate that CLA yolk levels off at a greater viscosity than control yolks, the statistical significance was not apparent from analysis of means. Viscosity decay rates were similar between control yolks, while CLA yolk viscosity decreased significantly more quickly in response to increasing temperature.

Egg yolks refrigerated for 20 days: Decreases in yolk initial viscosity seem to correspond to decreasing CLA concentration, but 0.9 mmole CLA yolks exhibited lower initial viscosity than 0.5 mmole CLA yolks. Control yolks, soy control yolks, and 0.9 mmole CLA yolks leveled off at similar viscosities, while all other CLA yolks displayed significantly greater asymptote values. Decay rates were similar among all yolk types. Correlations on viscosity and fatty acid profile from day 20 yolks revealed that viscosity was strongly correlated with CLA concentration ($R = 0.81$), total SFA ($R = 0.74$), palmitic acid concentration ($R = 0.72$), and had a strong inverse correlation with oleic acid concentration ($R = -0.72$). In addition, initial viscosity was moderately inversely correlated with EPA concentration ($R = -0.69$). It seems that as storage duration increases, CLA and saturated fat concentration were correlated with increasing yolk viscosity, while oleic and EPA concentration were negatively correlated with viscosity.

Egg yolks refrigerated for 30 days: Yolks containing 2.0 and 2.7 mmoles CLA exhibited nearly identical viscosity trends and have significantly greater initial viscosities than all other yolk types. Control, soy control, and 0.9 mmole CLA yolks have similar initial viscosities, while 0.5 mmole yolks exhibit significantly lower initial viscosity than all other yolk types. All viscosities level off at similar levels

regardless of CLA concentration. Multivariate analysis revealed only slight correlations between viscosity and fatty acid composition, namely, myristic acid ($R = 0.46$), palmitic acid ($R = 0.35$), CLA ($R = 0.36$), and a slight inverse correlation with DHA ($R = -0.38$).

In both control and soy control yolks, viscosity is similar during the first 10 days of refrigerated storage, and then a significant decrease is observed after 20 days refrigeration. Alternatively, the CLA-rich yolks have a significantly greater overall viscosity, and a marked decrease in viscosity is only apparent after 30 days of refrigeration. It may be that changes in viscosity after 30 days storage were more influenced by changes in other yolk quality parameters, such as moisture content and pH, rather than fatty acid composition.

CLA yolks containing the greatest CLA enrichment were considerably more viscous at low temperatures and retained their thickness for a longer storage duration. This effect may be advantageous in the preparation of mayonnaises and salad dressing recipes, where commercial products' microbial safety is more likely controlled by the acidity of the product, rather than a heat treatment. However, this increased viscosity was not solely a function of increased CLA concentration, but rather the distinct fatty acid composition changes that were a result of CLA enrichment. It was anticipated that the greater viscosities in CLA-rich yolks would be most highly correlated with saturated fatty acid content because a previous study identified significantly increased concentrations of saturated triacylglycerol species.¹⁶ However, a relationship between SFA and viscosity was only apparent after the yolks had been refrigerated for 20 days. It seems that yolk viscosity was a collective effect of the fatty acid composition, not just one specific type of fatty acid.

Yolks with most CLA enrichment had the greatest viscosity. The viscosity changes during refrigeration at 4°C indicate that CLA eggs that have been stored up to 20 days may provide improved food texture, mouth feel characteristics than fresh control eggs. This would enhance the viscosity of mayonnaises and salad dressings, custard desserts and sauces, which is the most desirable sensory attribute by consumers.²⁴

Egg Quality Analysis

Egg yolk physical properties: Table 5 shows whole egg weights of day 0 eggs, and eggs refrigerated for 20 and 30 days. Day 0 eggs showed the greatest variability in whole egg weight, so no

statistical differences could be determined. However, during refrigeration all egg weight variability decreased. Inclusion of control soy oil and CLA soy oil in the diet significantly reduced whole egg weights, relative to hens fed the unmodified control diet ($P = 0.009$), despite the additional metabolizable energy added with soy oil supplementation. Eggs containing the most CLA (2.7mmoles / 100 g yolk) had significantly lower whole egg weights after 20 and 30 days storage. However, all other CLA-rich egg weights were not significantly different from the soy control eggs ($p = 0.65$). Storage time did not have a significant effect overall on whole egg weights ($p = 0.98$). However, eggs produced from control soy oil or CLA-soy oil diets seemed to lose weight during storage while control yolks' total weight increased during storage. As an egg ages, it loses moisture and CO_2 through the shell pores, and both losses are dependent on storage temperature and relative humidity. While the decrease in total egg weight may be due to non-CLA soy fatty acids, CLA seemed to enhance this weight loss effect and may have some influence on how the egg is transpiring during storage. Other studies have reported conflicting results on total egg weight. Suksombat *et al.* (2006) reported significant egg weight decreases with CLA, while another study determined no difference among CLA and control yolk weights.^{5,8}

Table 5 shows the yolk weights of control and CLA-rich eggs as analyzed at 0, 20 and 30 days of storage. Egg yolks containing 2.0 mmoles of CLA were significantly smaller than soy control yolks ($p = 0.006$). However, all other CLA yolk weights were not significantly different from both control yolks. After 20 and 30 days of storage no significant differences could be determined among yolk types. CLA-rich soy oil prevented yolk weight reduction that has been previously reported from *cis*, *trans* CLA supplementation.⁵ There were no differences in yolk index (YI) at day 0 across in treatments, so YI was unaffected by CLA-rich soy oil supplementation ($p = 0.60$, Table 5). After 20 days there were no differences in yolk indices ($p = 0.73$). After 30 days of storage YI the only significant difference was that the 2.0 mmole CLA yolk YI were smaller than the controls. CLA-rich yolks have been previously reported to affect YI in comparison with control yolks.⁶ However, this study determined that changes in YI were more dependent on storage time than CLA content. In contrast, previous findings reported effect of CLA but did not account for normal storage time and temperatures. For example Kim *et al.* stored eggs at $-50^\circ C$, which may have altered YI, as yolks irreversibly gel during freezing.⁶ Schafer *et al.* observed very tall CLA yolks and larger YI than

control yolks but did not indicate egg storage time, which would produce very different results from those reported in our study.²⁷

Vitelline membrane strength: At day 0 VMS was greatest in control, soy control, and yolks containing 0.5 mmoles of CLA ($p < 0.001$, Table 5). After 20 days eggs with the highest CLA had the strongest VMS, while control yolks has the weakest VMS. After 30 days of refrigeration yolks containing 0.5 and 0.9mmole CLA had the strongest VMS, followed by 2.0 mmole CLA, standard control, and 2.7 mmole CLA yolks, while soy control yolks were still significantly weaker.

CLA incorporation maintained VMS up to 20 days, while diets containing unmodified soy oil resulted in weaker membranes after 5 days (data not shown). Vitelline membrane strength has been reported in omega-3 eggs, but not in previous CLA feeding trials. CLA-rich yolks with 2.7 mmole CLA did have significantly lower membrane strengths at day 0 and was maintained through day 20. After 30 days 2.7 mmole CLA yolks were significantly stronger than soy control eggs. Yolks containing 0.5 and 0.9 mmole CLA had the greatest VMS after 30 days of refrigeration. An omega-3 egg study determined that these designer eggs had significant decreases in vitelline membrane strength, resulting shorter shelf-life.²⁵ The fact that CLA yolks VMS increased during storage may be beneficial for shelf-life and storage.

Yolk pH and moisture content: Yolk pH was similar in all day 0 egg yolks at 6.2 (Table 5). After 20 days of refrigerated storage both types of control yolks, and the two lower CLA yolk concentrations had not changed significantly, while the 2.0 mmole and 2.7 mmole CLA yolks had pH values above 6.3. After 30 days in refrigeration control yolks and yolks containing on 0.5 mmole CLA had pH at 6.08 and 6.05, respectively, while the yolks containing greater levels of CLA had pH all above 7.15. These changes in pH were similar to previous yolk CLA enrichment reports.⁷

Table 10 shows changes in yolk moisture content during 30 days of refrigeration. Yolk moisture content was similar among day 0 eggs regardless of CLA concentration at 47-48%, and increased significantly in response to refrigerated storage ($p < 0.001$). This increase was significantly greater as yolk CLA concentration increased ($p = 0.008$). Yolks containing the three greatest CLA concentrations had moisture contents of 53% after 30 days of refrigeration, while both control and soy control yolks and 0.5 mmole CLA yolks had moisture contents slightly lower than 49%.

Both pH and moisture content increased with increased yolk CLA concentrations, which has been speculated to be due to increased permeability through the vitelline membrane.⁷ However, other CLA egg studies have reported some pink discoloration in the albumen after refrigeration, and have also attributed the effect to membrane permeability²⁶. This effect was not observed from *trans, trans* CLA eggs up to 30 days. Further investigations on the vitelline membrane physiochemical and structural properties may provide further insight into the aging and shelf-life of both CLA and standard eggs.

This study reported increase in saturated fatty acid in CLA eggs, relative to conventional eggs but not to the same degree as described in previous studies.^{1,2,5} Viscosity and VMS has not been previously reported in CLA eggs. Both determinations were greater than those of the control yolks and these qualities were preserved longer throughout storage. These enhanced qualities may benefit egg shelf-life and provide advantages in prepared egg based dressings and sauces. The study did not find changes in CLA yolk weights or yolk indices, relative to controls. In contrast, other studies report CLA eggs had smaller yolk weights and larger yolk indices relative to conventional eggs, but storage conditions were either unspecified or different from those in this study. Egg yolk viscosity and VMS in CLA eggs is worthy of further study as they may affect egg quality and have food processing applications.

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Tables and Figures

Table 1. Fatty acid composition of refined, bleached, deodorized (RBD) soy oil before photoisomerization, and resulting CLA-rich soy oil after 12 h photoisomerization.

Fatty acid	RBD Soy oil (g FA/ 100 g oil)	CLARSO (g FA/ 100 g oil)
Palmitic acid C16:0	12.2 ± 0.05	12.2 ± 0.02
Palmitoleic acid C16:1	0.09 ± 0.001	0.09 ± 0.001
Stearic acid C18:0	4.12 ± 0.04	4.24 ± 0.02
Oleic acid C18:1	25.0 ± 0.05	25.9 ± 0.06
Linoleic acid C18:2	55.0 ± 0.07	36.5 ± 0.05
Linolenic acid C18:3	3.54 ± 0.02	3.59 ± 0.02
<i>cis</i> -9, <i>trans</i> -11 CLA	ND ¹	1.64 ± 0.01
<i>Trans</i> -9, <i>cis</i> -11 & <i>cis</i> -10, <i>trans</i> -12 CLA	ND	2.14 ± 0.02
<i>trans</i> -10, <i>cis</i> -12 CLA	ND	1.27 ± 0.01
<i>trans, trans</i> CLA	ND	13.1 ± 0.02
Total CLA	ND	18.15 ± 0.03

¹Not detected

Table 2. Egg yolk total CLA concentration and polyunsaturated fatty acid concentrations from hens fed either a standard control diet, or a diet enriched with 10% soybean oil [N =120]. Soybean oils had 0% (soy control), 2.5%, 5%, 10% or 15% CLA concentrations. Concentrations are expressed as mmole of fatty acid per 100 g yolk. Connecting letters within the same row indicate significant differences in fatty acid concentration.

Yolk polyunsaturated fatty acids	Control yolk 0% CLA	Soy control yolk 0% CLA	0.5 mmole CLA (per 100 g yolk)	0.9 mmole CLA (per 100 g yolk)	2 mmole CLA (per 100 g yolk)	2.7 mmole CLA (per 100 g yolk)
<i>trans, trans</i> CLA	0 ± 0e	0 ± 0e	0.21 ± 0.01d	0.43 ± 0.02c	1.11 ± 0.06b	1.43 ± 0.18a
<i>cis-9, trans-11</i> CLA	0 ± 0e	0 ± 0e	0.14 ± 0.01a	0.15 ± 0.01a	0.13 ± 0.01a	0.15 ± 0.02a
<i>trans-9, cis-11 & cis-10, trans-12</i> CLA	0 ± 0e	0 ± 0e	0.08 ± 0.001d	0.17 ± 0.01c	0.31 ± 0.01b	0.41 ± 0.04a
<i>trans-10, cis-12</i> CLA	0 ± 0e	0 ± 0e	0.07 ± 0.01d	0.14 ± 0.01c	0.36 ± 0.02b	0.55 ± 0.06a
<i>cis-11, trans-13</i> CLA	0 ± 0e	0 ± 0e	0.01 ± 0.001d	0.03 ± 0.002c	0.07 ± 0.006b	0.11 ± 0.01a
Linoleic acid	16.97 ± 0.67b	28.03 ± 0.9a	27.46 + 0.53a	27.77 + 0.77a	27.51 + 0.66a	26.2 + 0.67a
Linolenic acid	0.31 ± 0.03c	0.87 ± 0.05a	0.81 + 0.04ab	0.78 + 0.05ab	0.77 + 0.03ab	0.69 + 0.06b
Arachidonic acid	0.17 ± 0.01a	0.13 ± 0.04a	0 + 0a	0 + 0a	0 + 0a	0.13 + 0.13a
Eicosapentanoic acid	1.75 ± 0.14ab	1.91 ± 0.05a	1.75 + 0.04ab	1.66 + 0.04ab	1.67 + 0.03ab	1.43 + 0.13b
Docosahexanoic acid	0.47 ± 0.01de	0.95 ± 0.04a	0.8 + 0.02b	0.69 + 0.01bc	0.6 + 0.01cd	0.45 + 0.05e
<i>Total PUFA</i>	19.68 ± 0.77b	31.9 ± 0.95a	31.34 + 0.55a	31.82 + 0.82a	32.54 + 0.71a	31.56 + 0.7a

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Table 3. Egg yolk total saturated and monounsaturated fatty acid concentrations from hens fed either a standard control diet, or diets enriched with 10% soybean oil [N= 120]. Soybean oils had 0% (soy control), 2.5%, 5%, 10% or 15% CLA concentrations. Concentrations are expressed as mmole of fatty acid per 100 g yolk. Connecting letters within the same row indicate significant differences in fatty acid concentration.

Yolk saturated fatty acids	Control yolk 0% CLA	Soy control yolk 0% CLA	0.5 mmole CLA (per 100 g yolk)	0.9 mmole CLA (per 100 g yolk)	2 mmole CLA (per 100 g yolk)	2.7 mmole CLA (per 100 g yolk)
<i>Myristic acid</i>	0.41 ± 0.01a	0.3 ± 0.01c	0.33 ± 0.01bc	0.39 ± 0.02ab	0.46 ± 0.03a	0.47 ± 0.02a
<i>Palmitic acid</i>	29.08 ± 0.38c	26.16 ± 0.33d	28.47 ± 0.23c	29.65 ± 0.36bc	30.67 ± 0.4ab	31.93 ± 0.65a
<i>Stearic acid</i>	11.55 ± 0.27c	12.52 ± 0.3c	14.97 ± 0.36b	16.19 ± 0.38ab	17.92 ± 0.38a	17.77 ± 0.74a
<i>Eicosanoic acid</i>	0.19 ± 0.18a	0.02 ± 0.01a	0.01 ± 0a	0.01 ± 0a	0.02 ± 0a	0.08 ± 0.05a
<i>Total saturated fatty acids</i>	41.22 ± 0.4c	38.99 ± 0.45d	43.77 ± 0.37bc	46.24 ± 0.51b	49.07 ± 0.42a	50.25 ± 1.3a
Yolk monounsaturated fatty acids	Control yolk 0% CLA	Soy control yolk 0% CLA	0.5 mmole CLA (per 100 g yolk)	0.9 mmole CLA (per 100 g yolk)	2 mmole CLA (per 100 g yolk)	2.7 mmole CLA (per 100 g yolk)
<i>Palmitoleic acid</i>	3.02 + 0.1a	1.48 + 0.03b	1.32 + 0.08bc	1.14 + 0.05cd	1.05 + 0.05cd	1.04 + 0.05d
<i>Oleic acid</i>	48.71 + 0.72a	40.01 + 0.63b	35.99 + 0.48c	33.35 + 0.66c	29.99 + 0.63d	30.12 + 1.26d
<i>Total monounsaturated fatty acids</i>	51.73 + 0.66a	41.49 + 0.64b	37.31 + 0.53c	34.48 + 0.65c	31.04 + 0.65d	31.16 + 1.29d

Table 4. Egg yolk viscosity variable estimates described by the exponential decay model, which includes R² value for each viscosity curve, initial viscosity at 4°C, asymptote (lowest part of curve) and average decay rate. Yolks were stored at 4°C prior to analysis. Variable estimates were compared by analysis of means at 0.05 alpha-level for each storage duration. Connecting letters within the same row indicate significant differences from the model mean.

	Control yolk	Soy control yolk	0.5 mmole yolk	0.9 mmole CLA yolk	2.0 mmole yolk	2.7 mmole yolk
Day 0 Egg yolks	<i>Model R² = 0.85</i>					
Asymptote (Pa·s)	0.09 ± 0.05b	0.24 ± 0.03a	0.27 ± 0.03a	0.2 ± 0.02a	0.21 ± 0.02a	0.28 ± 0.02a
Initial Viscosity at 4°C (Pa·s)	1.82 ± 0.04d	2.04 ± 0.04d	2.22 ± 0.05c	1.77 ± 0.06d	2.41 ± 0.05b	3.74 ± 0.07a
Decay rate	-0.04 ± 0.00a	-0.05 ± 0.003a	-0.06 ± 0.003b	-0.07 ± 0.005b	-0.07 ± 0.003b	-0.08 ± 0.002c
Egg yolks stored for 5 days	<i>Model R² = 0.93</i>					
Asymptote (Pa·s)	0.20 ± 0.02a	0.14 ± 0.02b	---	---	---	0.14 ± 0.02b
Initial Viscosity at 4°C (Pa·s)	1.87 ± 0.03b	1.58 ± 0.03c	---	---	---	3.01 ± 0.04a
Decay rate	-0.05 ± 0.002a	-0.04 ± 0.003b	---	---	---	-0.06 ± 0.002c
Egg yolks stored for 10 days	<i>Model R² = 0.82</i>					
Asymptote (Pa·s)	0.21 ± 0.02a	0.19 ± 0.03a	---	---	---	0.31 ± 0.02a
Initial Viscosity at 4°C (Pa·s)	1.80 ± 0.05b	1.77 ± 0.03b	---	---	---	3.20 ± 0.08a
Decay rate	-0.05 ± 0.004a	-0.05 ± 0.004a	---	---	---	-0.08 ± 0.003b
Egg yolks stored for 20 days	<i>Model R² = 0.82</i>					
Asymptote (Pa·s)	0.12 ± 0.02c	0.10 ± 0.02c	0.20 ± 0.02b	0.08 ± 0.02c	0.20 ± 0.02b	0.33 ± 0.02a
Initial Viscosity at 4°C (Pa·s)	0.72 ± 0.03c	0.80 ± 0.03c	1.45 ± 0.04c	1.17 ± 0.03b	2.52 ± 0.04a	2.86 ± 0.04a
Decay rate	-0.06 ± 0.007a	-0.05 ± 0.006a	-0.06 ± 0.004a	-0.05 ± 0.004a	-0.06 ± 0.002a	-0.07 ± 0.002a
Egg yolks stored for 30 days	<i>Model R² = 0.80</i>					
Asymptote (Pa·s)	0.08 ± 0.02a	0.5 ± 0.02a	0.04 ± 0.02a	0.09 ± 0.01a	0.07 ± 0.01a	0.07 ± 0.01a
Initial Viscosity at 4°C (Pa·s)	0.99 ± 0.03b	0.95 ± 0.02b	0.62 ± 0.04c	1.0 ± 0.02b	1.29 ± 0.02a	1.29 ± 0.02a
Decay rate	-0.05 ± 0.003a	-0.05 ± 0.003a	-0.05 ± 0.004a	-0.07 ± 0.003b	-0.06 ± 0.002b	-0.06 ± 0.002b
Effects Test	Prob > F					
Yolk Type	<0.0001*					
Storage	<0.0001*					
Yolk Type*Storage	<0.0001*					

Table 5. CLA-rich, soybean oil rich, and standard control egg yolk quality parameters were measured in quadruplicate on each storage duration. Connecting letters within the same column identify values with no significant difference at the 0.05 α -level.

Whole Egg Weight				Vitelline Membrane Strength			
Yolk Type	<i>0 Days in Storage</i>	<i>20 Days in Storage</i>	<i>30 Days in Storage</i>	Yolk Type	<i>0 Days in Storage</i>	<i>20 Days in Storage</i>	<i>30 Days in Storage</i>
Control	64.8 ± 2.31a	66.7 ± 2.1a	68.8 ± 0.6a	Control	5.28 ± 0.1a	3.96 ± 0.8ab	3.88 ± 0.1ab
Soy Control	62.7 ± 0.8a	62.3 ± 0.5b	62.3 ± 0.7b	Soy Control	5.34 ± 0.2a	2.68 ± 0.9b	2.08 ± 0.6b
0.5 mmole CLA	62.1 ± 3.68a	62.1 ± 1.9b	60.3 ± 0.6b	0.5 mmole CLA	5.18 ± 0.1a	3.40 ± 0.9ab	4.36 ± 0.7a
0.9 mmole CLA	61.4 ± 1.23a	59.3 ± 0.9b	58.6 ± 0.7b	0.9 mmole CLA	4.44 ± 0.1b	3.76 ± 0.6ab	4.74 ± 0.8a
2.0 mmole CLA	56.9 ± 1.27b	60.0 ± 1.1b	60.0 ± 1.5b	2.0 mmole CLA	4.20 ± 0.1b	3.28 ± 1ab	3.92 ± 1.0ab
2.7 mmole CLA	59.0 ± 0.83a	55.8 ± 0.4c	55.8 ± 0.6c	2.7 mmole CLA	4.46 ± 0.2b	4.9 ± 0.2a	3.48 ± 0.9ab
Effects Test	Prob > F			Effects Test	Prob > F		
Yolk Type	0.0094*			Yolk Type	0.58		
Storage	0.98			Storage	0.008*		
Yolk Type*Storage	0.36			Yolk Type*Storage	0.037*		
Yolk Weight				Yolk pH			
Yolk Type	<i>0 Days in Storage</i>	<i>20 Days in Storage</i>	<i>30 Days in Storage</i>	Yolk Type	<i>0 Days in Storage</i>	<i>20 Days in Storage</i>	<i>30 Days in Storage</i>
Control	18.7 ± 0.6ab	20.2 ± 1.8a	18.8 ± 0.17a	Control	6.21 ± 0.1a	5.96 ± 0.12ab	6.08 ± 0.01b
Soy Control	19.8 ± 0.4a	19.9 ± 0.8a	18.5 ± 0.74a	Soy Control	6.23 ± 0.1a	5.91 ± 0.15ab	6.31 ± 0.03b
0.5 mmole CLA	18.2 ± 1.1ab	18.9 ± 0.3a	18.1 ± 1.06a	0.5 mmole CLA	6.21 ± 0.2a	5.88 ± 0.07ab	6.05 ± 0.01b
0.9 mmole CLA	17.9 ± 0.4ab	18.7 ± 0.6a	18.3 ± 0.52a	0.9 mmole CLA	6.22 ± 0.1a	5.62 ± 0.02b	7.17 ± 0.35a
2.0 mmole CLA	16.0 ± 0.2b	19.0 ± 0.6a	17.9 ± 0.25a	2.0 mmole CLA	6.20 ± 0.05a	6.32 ± 0.1a	7.29 ± 0.09a
2.7 mmole CLA	17.2 ± 0.6ab	18.0 ± 0.7a	18.9 ± 0.49a	2.7 mmole CLA	6.20 ± 0.1a	6.43 ± 0.18a	7.5 ± 0.18a
Effects Test	Prob > F			Effects Test	Prob > F		
Yolk Type	0.0061*			Yolk Type	1		
Storage	0.038*			Storage	<0.0001*		
Yolk Type*Storage	0.27			Yolk Type*Storage	<0.0001*		
Yolk index				Moisture Content			
Yolk Type	<i>0 Days in Storage</i>	<i>20 Days in Storage</i>	<i>30 Days in Storage</i>	Yolk Type	<i>0 Days in Storage</i>	<i>20 Days in Storage</i>	<i>30 Days in Storage</i>
Control	0.4 ± 0.01a	0.35 ± 0.03ab	0.44 ± 0.01a	Control	48.1 ± 0.5a	47.4 ± 0.54b	46.4 ± 0.38c
Soy Control	0.4 ± 0.01a	0.36 ± 0.05ab	0.44 ± 0a	Soy Control	46.9 ± 0a	47.7 ± 0.62b	48.7 ± 0.38bc
0.5 mmole CLA	0.4 ± 0a	0.35 ± 0.03ab	0.45 ± 0.02ab	0.5 mmole CLA	47.5 ± 1.48a	48.4 ± 1.71ab	48.6 ± 0.06bc
0.9 mmole CLA	0.39 ± 0.01a	0.38 ± 0.01a	0.42 ± 0.01ab	0.9 mmole CLA	47.0 ± 1.56a	48.0 ± 1.06ab	50.6 ± 0.73ab
2.0 mmole CLA	0.4 ± 0.03a	0.37 ± 0.02ab	0.37 ± 0.01b	2.0 mmole CLA	47.5 ± 0.62a	50.0 ± 0.08ab	51.8 ± 0.86a
2.7 mmole CLA	0.38 ± 0.01a	0.34 ± 0.02b	0.42 ± 0.01ab	2.7 mmole CLA	47.2 ± 0.62a	51.4 ± 1.83a	53.2 ± 0.08a
Effects Test	Prob > F			Effects Test	Prob > F		
Yolk Type	0.48			Yolk Type	0.98		
Storage	< 0.0001*			Storage	<0.0001*		
Yolk Type*Storage	0.17			Yolk Type*Storage	0.008*		

CHAPTER 6. Isolation and characterization of chicken yolk vitelline membrane lipids using eggs enriched with conjugated linoleic acid

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Dear Ms. Shinn:

It is my pleasure to inform you that your manuscript entitled "Isolation and characterization of chicken yolk vitelline membrane lipids using eggs enriched with conjugated linoleic acid," Manuscript ID **LIPIDS-15-0177.R4**, is accepted for publication in *Lipids*.

Please send the final drafts of the text and tables in MS Word format and the figures in PDF format to Pam Landman, Production Editor and Peer Review Coordinator, at plandman@aoacs.org. All further correspondence regarding this manuscript should be directed to Pam Landman, Production Editor for *Lipids* at: AOCS Press, c/o AOCS, 2710 Boulder Dr., Urbana, IL 61802-6996 USA, Phone: +1-217-359-5401, ext. 124, Fax: +1-217 -351-8091.

On behalf of the Editorial Board I congratulate you. We appreciate your consideration of Lipids for publication of your research and look forward to future submissions from your group.

My best regards,

Eric

Dr. Eric Murphy

Editor, Lipids

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Abstract

The vitelline membrane (VM) encloses the chicken egg yolk, separating it from albumen. VM weaken during storage, and dietary lipid modification significantly affects its strength. However, no studies characterize the fatty acyl residue (FA) composition of VM, and reports of VM isolation and quantified lipid content are inconsistent. Therefore, the objectives of this study were 1) To develop a washing and isolation method that removes residual yolk from VM without damage 2) To determine the FA and lipid composition of CLA-rich egg yolk VM, relative to controls 3) To determine the effect of 20 days refrigeration on VM FA and lipid composition. To determine VM FA and lipid composition, thirty-six hens received either: a corn-soybean meal-based control diet ('Control'), or the Control supplemented with either 10% soy oil ('Soy control'), or 10% CLA-rich soy oil ('CLA') for 30 days. VM were analyzed the day of collection ('fresh'), or after 20 days of refrigeration ('refrigerated'). There were no differences in FA compositions of fresh and refrigerated membranes within a treatment. CLA-rich yolk VM contains: CLA, greater SFA, and significantly greater DHA relative to controls. Direct MALDI-TOF-MS identified 15 phosphatidylcholines, 3 phosphatidylethanolamines, 1 sphingomyelin, and 15 triacylglycerols in VM. Lipid species that showed significant differences among egg types included 9 phosphatidylcholines and 6 triacylglycerols. MALDI analysis indicated significant differences in 9 lipid classes on the VM inner layer. After refrigeration, 5 lipid classes on the inner layer and 7 lipid classes on the outer layer had statistically significant differences among VM types.

Keywords: Egg yolk vitelline membrane – conjugated linoleic acid – sample preparation – SEM-Fatty acyl residue composition -- Direct MALDI-TOF MS

Abbreviations

CLA - Conjugated linoleic acid

d - Days

FA - Fatty acyl residue

FAME - Fatty acid methyl ester

GC-FID - Gas chromatography-flame ionization detector

MALDI-TOF MS – matrix-assisted laser desorption ionization time of flight mass spectrometry

MUFA - Monounsaturated fatty acyl residue

PUFA - Polyunsaturated fatty acyl residue

SFA - Saturated fatty acyl residue

VM – Vitelline membrane

Introduction

The vitelline membrane (VM) surrounds the chicken egg yolk, separating yolk from the albumen. It has two fibrous layers: a glycoprotein layer that is in contact with the albumen, and a lipoprotein fibrous layer that is in contact with the yolk [1]. Between the two fibrous layers is a granular continuous membrane of unreported composition [1, 2]. The VM strength and integrity is essential in preventing yolk contamination of the albumen. This is important as even minimal yolk contamination will decrease albumen foaming volume by 70% [3].

Reports of VM lipid content, as determined by proximate analysis, are inconsistent [1, 2, 4]. While VM proteins have been identified [1, 5], the VM lipid composition is not known. VM lipid quantification was originally performed by washing the membrane using 1:3 (v/v) ether:ethanol followed by a water wash [2]. The membranes were then dried and lipids extracted using chloroform:methanol (1:1, v/v) for 48 h, and the lipid content was found to be 3.2%, dry basis [2]. However, this method probably greatly underestimated total lipid content, as ether and ethanol removed lipids from the membrane prior to quantifying lipid content.

Trziska & Smolinska [4] cited the VM washing and extraction method of Bellairs [2], but changed the ratio of ether:ethanol (v/v) from 1:3 to 3:1 and reported 5% membrane lipids. In addition, these authors reported isolating VM with an undefined series of saline and distilled water washes, which may have left residual yolk on the membrane, and then extracted lipids using chloroform:methanol, resulting in 13.2-15.9% lipids [4]. An additional study determined the VM lipid content to range from 3 -10% after water washing, drying, and Soxhlet extraction of the membranes [6]. Water washing may have resulted in residual yolk on the membrane, producing an overestimation of total lipids. In addition, the Soxhlet extraction may provide inaccurate results with such small lipid content samples due to lipid oxidation and adsorption on glassware [7].

The studies above showed that it is likely that water washing may not remove residual yolk from the VM, while the use of solvents may disrupt the membrane and extract membrane lipids that are not included in the total lipid analysis. Original Scanning electron microscopy (SEM) images of the VM were published in Kido & Doi [1]. In the present study SEM was employed to observe any residual yolk or membrane disruption due to solvent effects during washing. Using the images will aid in choosing a suitable washing method to be selected that removes yolk lipids without damaging the membrane. Subsequently, VM lipids could be accurately characterized.

The strength of the vitelline membrane in chicken eggs naturally decreases during refrigeration [8,9]. Some factors that have been reported to affect the VM strength include: increased osmotic pressure from water migration into the yolk [10] and physical changes such as the network of fibers of the VM disappearing during storage [8]. Furthermore, VM strength is significantly less in eggs enriched with omega-3 fatty acyl residues [11]. In contrast, conjugated linoleic acid (CLA) enriched eggs maintain VM strength longer during 20 days of refrigeration than do commercial control eggs [12]. This could be attributed to the higher saturated fat content, because if there was greater saturated fat in the membrane, it may become less fluid, as CLA-rich yolks haven been determined as more rubbery [25]. Both omega-3 and CLA-enriched eggs are produced by modifying the hen's dietary fat composition. However, no studies have determined the fatty acyl residue (FA) composition of this membrane or described the intact lipid species in VM. This information would be useful in determining why CLA enriched eggs have stronger VM.

The traditional approach would be to extract lipids from isolated VM, hydrolyze and derivatize them into fatty acid methyl esters (FAMES), and determine FA composition by GC-FID. In addition, matrix assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS) has been previously used to characterize proteins in biological membranes [13], and more recently has been used to compare the lipid species in CLA-rich and control egg yolks [14]. This method can be used without prior lipid extraction to determine the lipid species present in the membranes, as well as the possible differences between CLA-rich and control vitelline membranes.

Therefore, the objectives of this study were to 1) To develop a VM washing and isolation method that will remove residual yolk without damaging the VM 2) To determine the FA and lipid composition of

CLA-rich egg yolk VM, relative to control VM and 3) To determine the effect of 20 days of refrigeration on VM lipid composition in CLA-rich and control yolks.

Materials and Methods

Dietary Treatments and Egg Production

Refined, bleached, deodorized soybean oil used for CLA-rich soybean oil production and feed preparation was provided by Riceland Foods (Stuttgart, AR). A CLA-rich soybean oil containing 15% CLA in triacylglycerol form was produced by linoleic acid photoisomerization using a pilot scale unit and method of Jain & Proctor [15]. The feed used in this study was a pelleted commercial corn and soybean meal-based broiler finisher diet (Cobb-Vantress, Siloam Springs, AR). Three dietary treatments were used in this study: 1) unmodified commercial feed ("Control"), 2) Control + 10% soy oil ("Soy control") or 3) Control + 10% CLA-rich soy oil ("CLA"). Soy control and CLA diets were prepared by adding 10% (wt.) soy oil to the standard commercial feed and combined using a Hobart stand mixer. The contents of these three diets are presented in Table 1. It should be noted that the commercial diet used was formulated for meat-type chickens, making it markedly deficient in calcium, a critical nutrient for laying hens. Although this was not the ideal feeding regiment for layer chicken eggshell quality, it still allowed determination of dietary fatty acids on vitelline membrane composition. In this short-term study we did not observe any issues with egg shell quality, although it may have become apparent after a longer feeding duration. Furthermore, by adding 10% of either soy oil or CLA-rich soy oil to commercial feed to create the experimental diets, the nutrient content of the original diet was diluted by 10%.

One hundred single-comb White Leghorn chicks were reared under standard commercial conditions. At 35 weeks, thirty-six hens were randomly assigned to single bird cages in blocks of 6 birds each, separated by 2 empty cages. All hens received the Control diet until the feeding trial began at 36 weeks. Then each diet (Control, Soy control, or CLA) was randomly assigned to 2 blocks of 6 birds each, resulting in 12 birds receiving each treatment diet. Animal procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee (Protocol # 13033).

Hens were fed *ad libitum* for a total of 30 days. Control eggs collected in the first 20 days of feeding were used for *Experiment 1: Vitelline membrane washing and isolation*. After 20 days of

treatment feed administration, which allows maximum CLA accumulation in the egg [17,18], eggs from each treatment group were collected daily for 10 days, and labeled with cage number and date, and used for *Experiment 2: Vitelline membrane FA and lipid composition*. Eggs collected on days 1-5 of the 10 day collection period were analyzed as “fresh.” These eggs were stored in single layer flats at 4°C for 6 h prior to subsequent analysis. Eggs collected on days 6 - 10 were refrigerated for 20 days at 4°C before subsequent analysis. In summary, duplicate sets of membranes from each hen and storage condition (fresh or refrigerated) were analyzed for fatty acyl residue composition. In addition, triplicate sets (from different collection days) of 6 randomly selected membranes from each treatment group and storage condition were analyzed for lipid composition by direct MALDI-TOF-MS. Due to availability of the MALDI-TOF, only 6 membranes were able to be analyzed from the three selected collection days for fresh and refrigerated eggs.

Experimental Design

Experiment 1: Vitelline membrane washing and isolation

All chemicals used for membrane washing and isolation were analytical grade. All chemicals used for SEM preparation and imaging were microscopy grade.

Washing Protocols: Eight sets of 6 Control egg yolks each were separated from albumens and rolled on a *Kimwipe* to remove residual adhering albumen. Each VM was punctured using a scalpel and yolk was drained from the VM. Duplicate sets of VM were subjected to one of the following washing methods:

- 1.) VM washed with deionized water using a nozzle to thoroughly wash the punctured membrane.
- 2.) VM set was washed in 50 mL ether:ethanol 1:3 (v/v), followed by a water wash [2]
- 3.) VM set was washed in 50 mL ether:ethanol 3:1 (v/v), followed by a water wash [4]
- 4.) VM washed in distilled water with 1% NaCl [4]

VM lipid extraction: Washed membranes sets from each method were dried under vacuum for 24 h and weighed. VM lipid extractions were performed using the Folch method [19]. Lipid content was quantified gravimetrically.

Fatty acyl residue analysis of VM lipids and yolk: This was performed to determine if the fatty acyl residue (FA) composition of the VM from each washing treatment differed from that of yolk, or if there was suspect residual yolk due to the washing method. The duplicate lipid samples extracted from the washed VM (all treatments) were derivatized to fatty acid methyl esters (FAMES) using a rapid, micro FAMES preparation method [20]. In addition, duplicate sets of 3 egg yolks were prepared for FAMES analysis by separating and homogenizing the yolks in a set, extracting the lipids [19] and FAMES conversion [20]. Duplicate FA profiles for VM and yolk samples were obtained for each FAME preparation by GC with a SP 2560 fused silica capillary column (100m x 0.25 mm i.d. x 2 µm film thickness; Supelco Inc. Bellefonte, PA), and a flame ionization detector (FID model 3800, Varian, Walnut Creek, CA). Samples of 3.0 µL were injected by autosampler. The FID settings were as follows: split ratio 1:100; oven temp = 250°C; sensitivity = 12, He gas = 30 mL/min, H₂ = 31 mL/min, air = 296 mL/min, and over program time = 111 min. Total FA composition was calculated with the following equation:

$$[\% \text{ fatty acyl residue}] = ([\text{HME}] \times \text{sample peak area} \times \text{relative response factor}) / \text{HME peak area.}$$

Statistical analysis: Lipid extract weights from each washing procedure were analyzed by ANOVA, and compared using a Tukey's HSD test with a 0.05 alpha level. FA compositions of VM lipids extracted and those extracted from egg yolk were also analyzed by ANOVA, and compared using a Tukey's HSD test with a 0.05 alpha level.

Vitelline membrane SEM imaging: Triplicate membranes from each washing method were prepared for SEM using the following standard protocol: Each membrane set was immersed in approximately 10 mL Karnovsky's fixative for 120 minutes. Membranes were then washed 2x with 0.05M cacodylate buffer (Personal communication, Arkansas Nano & Bio Materials Characterization Facility). Samples were dehydrated using a 30%, 50%, 70%, 80% and 90% ethanol series, then spread out flat in a petri dish and allowed to dry under vacuum for 3 h. Completely dry, flat membrane sections were applied to studs and sputter coated (EMI Tech Sputter Coater SC7620). Images of both the outer and inner surfaces of the VM were taken at 5000x magnification with a FEI Nova Nanolab SEM.

Experiment 2: Vitelline membrane FA and lipid composition

FAMES analysis: All lipid extraction solvents were analytical grade. The FAME internal standard heptadecanoic acid (C17:0) used was purchased from Supelco (Bellefonte, PA).

Duplicate sets of twelve VM from each dietary treatment were isolated using the deionized water washing method (Method 1). This was performed on both fresh and refrigerated samples. The washed membranes were dried under vacuum for 24 h and weighed. VM lipid extractions were performed using the Folch method and lipid content was quantified gravimetrically [19]. The duplicate lipid samples from each treatment were converted into FAMES by method of Lall et al. [20] for subsequent FA composition analysis by GC-FID in duplicate. Total FA composition, CLA concentration and CLA isomer concentration were calculated as described in Experiment 1. The position of double bonds in *trans,trans* CLA-rich soybean oil produced by photoisomerization [15] were previously determined by silver ion HPLC [21].

Lipid analysis: The inner and outer surfaces of fresh and refrigerated vitelline membranes from each treatment group were analyzed by direct MALDI-TOF-MS analysis. Six clean, isolated membranes portions (approximately 10x10 mm) were applied to conductive microscope slides (ITO coating, part No. 237001, Bruker Corporation) with either the inner or outer membrane exposed for MALDI analysis. One molar 2,5-Dihydroxybenzoic acid (DHB) in 90% MeOH, 10% water containing 0.1% trifluoroacetic acid was used as the MALDI matrix. This was applied in 2 μ L aliquots over each membrane, until DHB matrix crystals were visually observable on the membranes and covered the portion completely. After complete drying under vacuum, slides were fitted to a MALDI target slide holder (Bruker Corporation). The slides were imaged by an Ultraflex II MALDI-TOF/TOF with a 200 Hz smart beam laser in the reflector mode. The extraction voltage was set to 25 kV, and matrix suppression was set to $m/z < 200$. Data were acquired using FlexControl (FC 3.0) and FlexImaging 2.0 software under Compass 1.2 settings (Bruker, Billerica, Massachusetts). Twenty spectra from each set of 6 membrane and treatment group were collected. This experiment was repeated in triplicate. The data was processed using Bruker FlexImaging 2.0, Flex Analysis 2.4/3.0, and ClinProTools 2.2 software (Bruker, Billerica, Massachusetts). Lipid species were identified in accordance with the reports of CLA-rich and control yolk lipids [14,22]. This was performed by submitting both low- and high-resolution monoisotopic m/z peaks, and the FA composition information, which was obtained from FAMES analysis, to the www.lipidmaps.org database.

Statistical analysis: Vitelline membrane FA composition was compared by JMP 11.0 software using 2-way ANOVA to determine the effects of yolk type and storage using Tukey's HSD with and 0.05 alpha level. The lipid intensities were compared in ClinPro Tools 2.2 software to determine significance differences with and 0.05 alpha level.

Results

Experiment 1: Vitelline membrane washing and isolation

Vitelline membranes isolated by each washing method and visualized by SEM are shown in Figure 1. In addition, Table 2 reports the amount of lipid extracted from VM after each washing protocol and the resulting FA composition from GC-FID analysis. The first panel in Figure 1 visualizes the inner and outer VM layers that were isolated by simply washing the membrane thoroughly with deionized water dispensed through a nozzle (Washing Method 1). The outer fibrous layer appears intact and the image is similar to those obtained by Kido & Doi [1] but with much better resolution. The inner layer is smooth and appears to have pores on the surface. Figure 2 shows a 150,000 magnification of the pores apparent on the inner VM layer. These pores have a range of 40 to 150 nm. These are the first reported images of VM inner layer pores.

Using Washing method 1 before lipid extraction resulted in 4% lipid content. This lipid content is within the range of lipid contents that were previously reported (2-15%) [1,4]. Both surfaces of the membrane appear undamaged and their integrity intact. Membrane appearance matched in subsequent replications of SEM VM preparations after washing method 1. The FA composition of VM isolated by Method 1 does not match the FA composition of extracted yolk lipids (Table 2). This along with the visual evidence from SEM indicates that there was no visible residual yolk left on the membrane that may confound subsequent FA and lipid composition analysis.

The second panel in Figure 1 are images of inner and outer VM washed with ethyl ether:ethanol 1:3 (v/v), followed by a water wash, repeating the protocol of Bellairs [2]. The outer fibrous layer seems intact, however the pores on the inner layer appear enlarged and the whole surface appears physically altered, most likely by solvent interaction from the wash. This isolation procedure resulted in 9.4% lipid yield from the VM. The solvents used seemed to disrupt the outer membrane, and not fully isolate VM

from residual yolk, resulting in greater lipid content than VM from Washing Method 1. In addition, there are no significant differences in the FA composition of the VM isolated by Method 2 and that of extracted native yolk lipids.

The third panel in Figure 1 shows a VM that seems even more altered, likely due to the greater concentration of ether used in washing method 3 which replicated the washing protocol of Trziska & Smolinska (ethyl ether:ethanol 3:1 (v/v)) [4]. This washing protocol resulted in only 1.9% lipids extracted from VM. The solvents used seemed to disrupt both the inner and outer layers of the membrane. It is very likely that this washing method extracted membrane lipids that would then not be included in subsequent membrane lipid analyses. More evidence of this is shown in Table 2 where the FA composition is dissimilar from yolk FA. In addition, 20:5n-3 and 22:6n-3 are not detected from these membrane extractions, but these FA are well known as structural membrane lipids [23] and are likely present in this biological membrane.

The bottom panel in Figure 1 shows inner and outer VM layers washed with 1% NaCl in water, which resulted in 15% lipid extraction from the membrane. This lipid content corresponds with that of Trziska & Smolinska using the same isolation method [4]. However, the outer layer appears to have a significant amount of residual albumen present, as well as residual yolk on the inner layer. It is likely that the yolk residue present resulted in a greater lipid extract yield, and would not be suitable for subsequent VM lipid analysis. Furthermore, the FA composition of VM isolated by Method 4 is identical to the FA of yolk.

Based on these findings, VM isolation for subsequent analysis was best obtained by Washing Method 1 - Thoroughly washing the membrane with slightly pressurized deionized water. This preparation step will allow subsequent membrane lipid extraction, fatty acyl residue analysis by GC-FID, and lipid analysis by direct MALDI-TOF-MS.

Experiment 2: Vitelline membrane FA and lipid composition

FAMES analysis: The FA composition of each VM from each treatment yolk is reported in Table 3.

Firstly, CLA was incorporated into the VM as 1.3% of total FA, with *trans*-9, *trans*-11 and *trans*-10, *trans*-12 CLA as the most abundant isomers. The non-conjugated linoleic acid (18:2) concentration is significantly greater in the Soy control VM at 19.1% of total FA, followed by CLA at 15.8 %, which are

both significantly greater than Control VM at 10.8 %. This increase in C18:2 corresponds to the much greater levels available in the Soy control and CLA-rich diets. In previous CLA yolk accumulation studies the total LA (CLA + LA) was not significantly different among CLA and Soy control yolks [12, 14, 18]. However total LA accumulation in VM is significantly lower in CLA-rich VM than Soy control VM.

The saturated FA present in the membrane were palmitic (16:0) and stearic acid (18:0). Palmitic acid was significantly lower in Soy control VM at 32%, while 16:0 in the Control and CLA VM 16:0 did not significantly differ at 34% and 35% respectively. However, 18:0 content in CLA VM was significantly greater at 13.2% of total FA, compared to both Control and Soy control VM, which contained 9.0% and 9.4% stearic acid respectively. In previous CLA-yolk accumulation studies saturated FA concentration of both 18:0 and 16:0 have been reported as significantly greater in CLA-rich yolks [12,14,17,18]. However, only a significant increase in 18:0 concentration was found in the vitelline membrane. CLA has been shown to inhibit stearyl-CoA desaturase, preventing the conversion of 18:0 to 18:1, which can in turn affect membrane fluidity [26].

The monounsaturated fatty acids (MUFA) present in VM are palmitoleic (16:1) and oleic acid (18:1). Palmitoleic acid concentration was significantly greater in Control VM at 2.6%, than both Soy control and CLA VM at 1.5% and 1.1%, respectively. The subsequent decrease in MUFA as a result of CLA or control soy oil FA accumulation has been previously reported [12,14,17,18]. The longer chain PUFA detected in VM are linolenic acid (18:3), arachidonic acid (20:4n-6), and docosahexaenoic acid (DHA, 22:6n-3). The 18:3 VM concentrations are not significantly different among the three treatment groups. The AA concentration is significantly greater in CLA VM versus both controls, which were not significantly different from one another. The DHA concentration is greatest in Soy control VM, which is significantly greater than Control VM, but not CLA VM. The FA composition of refrigerated VM were also determined. However, there were no significant differences in FA composition between fresh and refrigerated VM.

Lipid analysis: Although no changes in the FA composition of the membrane were detected after refrigeration, direct MALDI-TOF-MS analysis of the intact membranes allowed further comparison of lipid species present within and among treatment VM.

MALDI-TOF-MS analysis identified 15 phosphatidylcholine (PtdCho), 3 phosphatidylethanolamine (PtdEtn), 1 sphingomyelin (SM), and 15 triacylglycerols (TAG), all of which have been previously identified in other egg yolk MALDI-TOF-MS studies [14,22]. Of these, the lipid species that showed significant differences in either fresh or refrigerated eggs among treatments are listed in Table 4 including: 9 PtdCho species and 6 TAG.

In fresh membranes, the significant differences in PtdCho and TAG species are seen on the inner membrane. Seven PtdCho species were detected at significantly greater intensities in CLA inner VM, in respect to both Control VM, as were 3 TAG species. Mass spectra of these consistently different intensities are included in Figure 3.

The MS intensities for 20 d refrigerated membranes are also presented in Table 4. The inner membrane only showed significant differences in 5 TAG species among treatment groups. In addition, stored VM showed PtdCho intensities that differed only on the outer membrane layer, while no PtdCho differences could be detected on the inner VM, Figure 3. These differences were apparent and statically significant in all three replications of the experiment.

Discussion

This is the first report of vitelline membrane lipid modification in response to changes in the chicken's dietary lipids. The VM fatty acyl residue composition of chicken yolks includes CLA, greater SFA and significantly greater DHA as a result of CLA accumulation in the egg, relative to the standard control egg. It has been reported that omega-3 rich eggs have significantly weaker membranes [11], while CLA-rich eggs maintain their strength [12]. While there were no obvious changes in FA composition among refrigerated and fresh VM that could solely explain a CLA-rich membrane maintaining strength, there are definite modifications in the VM lipid composition that may be affecting how the membrane ages or oxidizes during the typical shelf-life of an egg. Understanding how the VM ages, and how that can be modified by simple dietary adjustments may provide a means to develop more shelf-stable, longer lasting eggs to the marketplace.

This is also the first report of using MALDI-TOF-MS to determine lipid species in chicken egg vitelline membranes. It is interesting to note that in fresh eggs, the major VM lipid differences were seen

in the inner layer of the VM, while stored eggs had more significant differences in lipid species on the outer VM layer. This report does support the idea that there are lipid composition modifications in the VM during storage, and this seems to have substantial effects on VM strength and integrity.

In addition, changes in the VM lipid composition detected on the inner and outer membrane layers by direct MALDI-TOF-MS would not be apparent through traditional FAMES analysis by GC-FID. This research is a proof of concept that direct MALDI-TOF MS can be an invaluable tool for the identification of lipid species in biological membranes, without the need for lipid extraction and FAME derivation. While the changes in intact lipid species composition detected by direct MALDI-TOF-MS do not solely explain why egg yolk vitelline membranes general weaken during refrigeration, or why CLA-rich yolks maintain their VM strength, this analysis allowed a real time look at a biological membranes' lipid composition, without the need of lipid extraction. This use of direct MALDI-TOF-MS to both inner and outer vitelline membranes show how applicable the technique is to accurate identification of intact lipid species, with less sample prep, and no need for prior lipid extraction.

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Tables and Figures

Table 1. Fatty acid (FA) composition and calculated nutrient contents of the hen's diets. The three dietary treatments used were: 1) unmodified commercial feed ("Control diet"), 2) Control + 10% soy oil ("Soy control diet") or 3) Control + 10% CLA-rich soy oil ("CLA diet"). Soy control and CLA diets were prepared by adding 10% (wt.) soy oil to the standard commercial feed and combined using a Hobart stand mixer.

	Control diet	Soy control diet	CLA diet
Crude Fat (g/kg)	33.9 ± 3.0	130 ± 2.0	130 ± 2.0
FA composition (g/kg feed)			
Palmitic acid C16:0	4.5 ± 0.03	16.5 ± 0.03	16.4 ± 0.01
Stearic acid C18:0	1.8 ± 0.11	5.9 ± 0.04	6.0 ± 0.01
Oleic acid C18:1	17.1 ± 0.16	42.1 ± 0.04	42.4 ± 0.04
Linoleic acid C18:2	10.2 ± 0.12	64.4 ± 0.03	44.2 ± 0.03
Linolenic acid C18:3	0.2 ± 0.09	3.7 ± 0.02	3.8 ± 0.01
<i>cis</i> -9, <i>trans</i> -11 CLA	ND ¹	ND	1.6 ± 0.01
<i>Trans</i> -9, <i>cis</i> -11 & <i>cis</i> -10, <i>trans</i> -12 CLA	ND	ND	2.1 ± 0.01
<i>trans</i> -10, <i>cis</i> -12 CLA	ND	ND	1.3 ± 0.007
<i>trans</i> -9, <i>trans</i> -11 and <i>trans</i> -10, <i>trans</i> -12 CLA	ND	ND	14.9 ± 0.01
Total CLA	ND	ND	19.8 ± 0.02
Metabolisable Energy (kcal/kg)	2761	3661	3661
Crude Protein (%)	15.4	13.9	13.9
Calcium (%)	1.45	1.31	1.31
Phosphorous (%)	0.43	0.38	0.38
Sodium (%)	0.15	0.13	0.13
Linoleic Acid (%)	1.02	64.1	64.0
<u>Amino Acids</u>			
Lysine (%)	0.61	0.55	0.55
Methionine (%)	0.27	0.24	0.24

Table 2. Control Egg yolk lipids and VM lipids extracted from each washing method were quantified, and subsequently analyzed as FAMES by GC-FID

Vitelline membrane FA composition					
	Yolk	Washing Method 1	Washing Method 2	Washing Method 3	Washing Method 4
<i>Lipids Extracted (% sample)</i>	31.2 ± 1%	4.3 ± 0.5% ^c	9.4 ± 0.2% ^b	1.9 ± 0.8% ^d	15.0 ± 0.5% ^a
FA (% total)		mole %			
16:0	29.0 ± 0.1 ^b	32.3 ± 0.6 ^a	29.1 ± 0.2 ^b	23.8 ± 0.1 ^c	28.9 ± 0.2 ^b
16:1	3.0 ± 0.2 ^a	2.47 ± 0.2 ^b	3.1 ± 0.3 ^a	1.0 ± 0.1 ^a	3.4 ± 0.3 ^a
18:0	12.6 ± 0.3 ^a	9.34 ± 0.4 ^b	12.2 ± 0.2 ^a	9.9 ± 0.3 ^b	12.5 ± 0.2 ^a
18:1	48.5 ± 0.4 ^a	42.9 ± 0.7 ^b	48.0 ± 0.1 ^a	42.1 ± 0.9 ^c	48.1 ± 0.4 ^a
18:2	16.9 ± 0.2 ^b	11.1 ± 0.8 ^c	17.1 ± 0.2 ^b	22.9 ± 0.5 ^a	17.0 ± 0.3 ^b
18:3n-3	0.3 ± 0.05 ^b	1.41 ± 0.1 ^a	0.3 ± 0.04 ^b	0.3 ± 0.04 ^b	0.3 ± 0.06 ^b
20:5n-3	1.75 ± 0.1 ^a	0.12 ± 0.02 ^b	1.75 ± 0.2 ^a	ND	1.73 ± 0.1 ^a
22:6n-3	0.5 ± 0.04 ^a	0.2 ± 0.06 ^b	0.5 ± 0.03 ^a	ND	0.5 ± 0.04 ^a

1. VM washed with deionized water using a nozzle to thoroughly wash the punctured membrane.
2. VM washed in 50 mL ethyl ether:ethanol 1:3 (v/v), followed by a water wash [2].
3. VM washed in 50 mL ethyl ether:ethanol 3:1 (v/v), followed by a water wash [4].
4. VM washed in distilled water with 1% NaCl [4]

Figure 1. Vitelline membrane inner and outer layers were imaged by SEM to assess possible damage to the layers after each washing protocol.

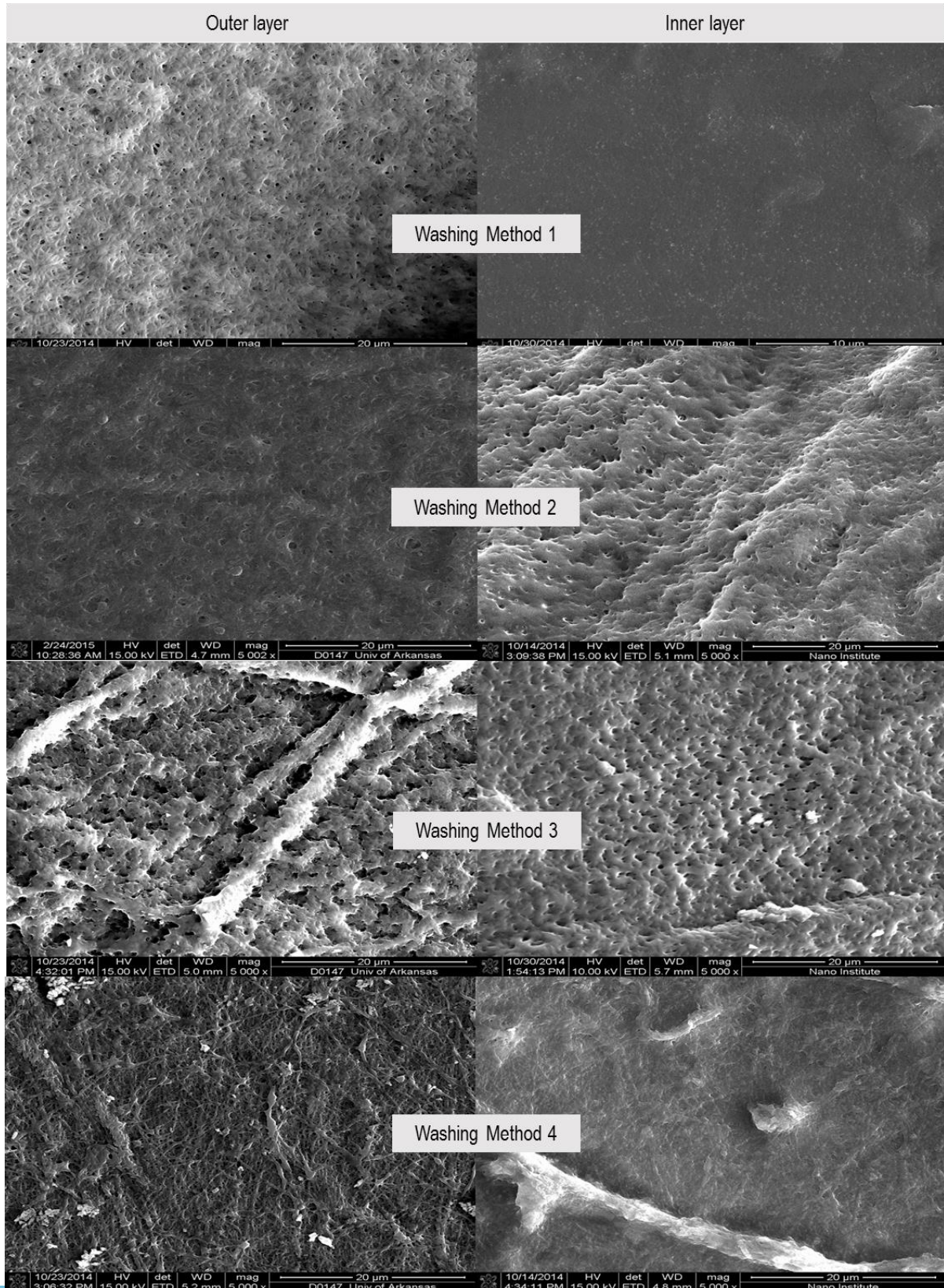


Figure 2. Vitelline membrane inner layer pores. Pores present range from 40 to 150 nm.

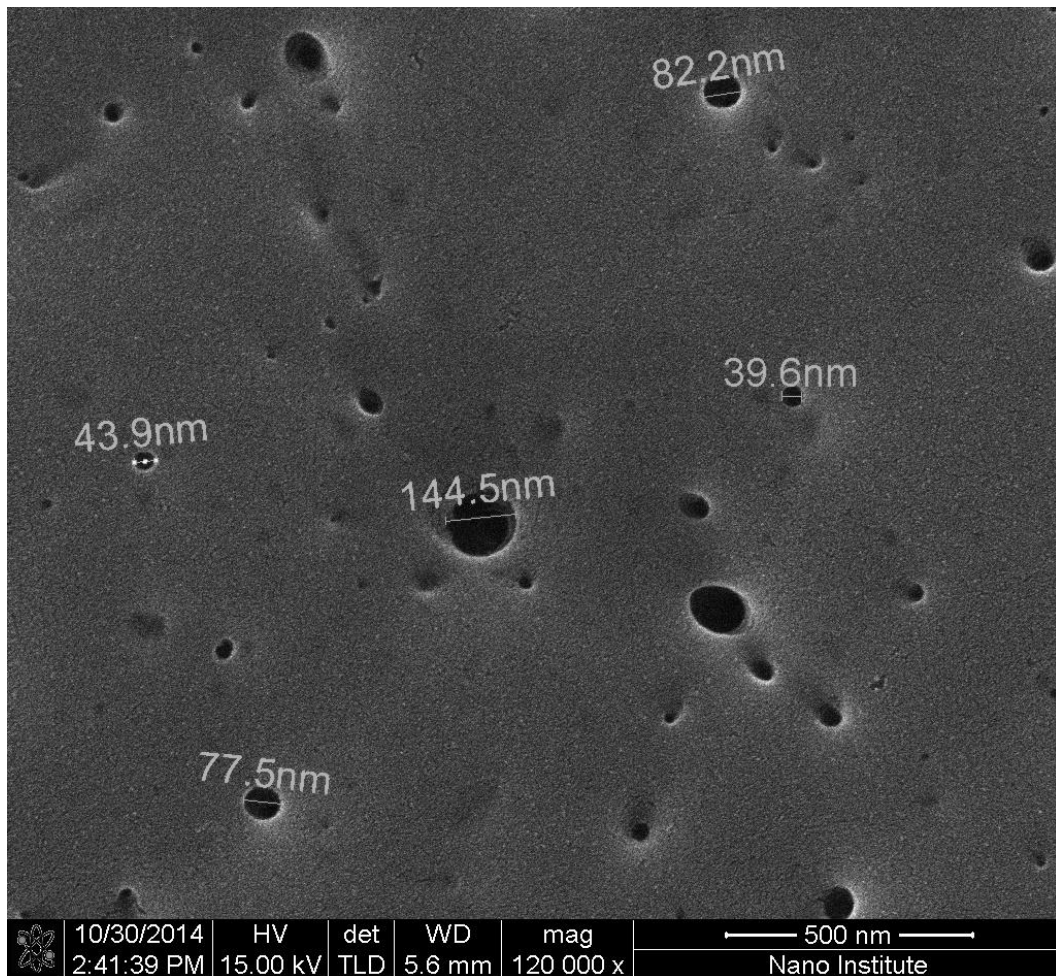


Table 3. Chicken egg yolk vitelline membrane FA composition (mol %) from Control eggs, CLA-rich eggs, and Soy control eggs. Lipid extraction and subsequent FA analysis were performed on duplicate sets of 12 membranes from each treatment egg group on both fresh and refrigerated VM. Connecting letters indicate significant differences at the 0.05 α -level. There were no significant differences in the FA profile between fresh and refrigerated VM within a treatment group ($p < 0.001$).

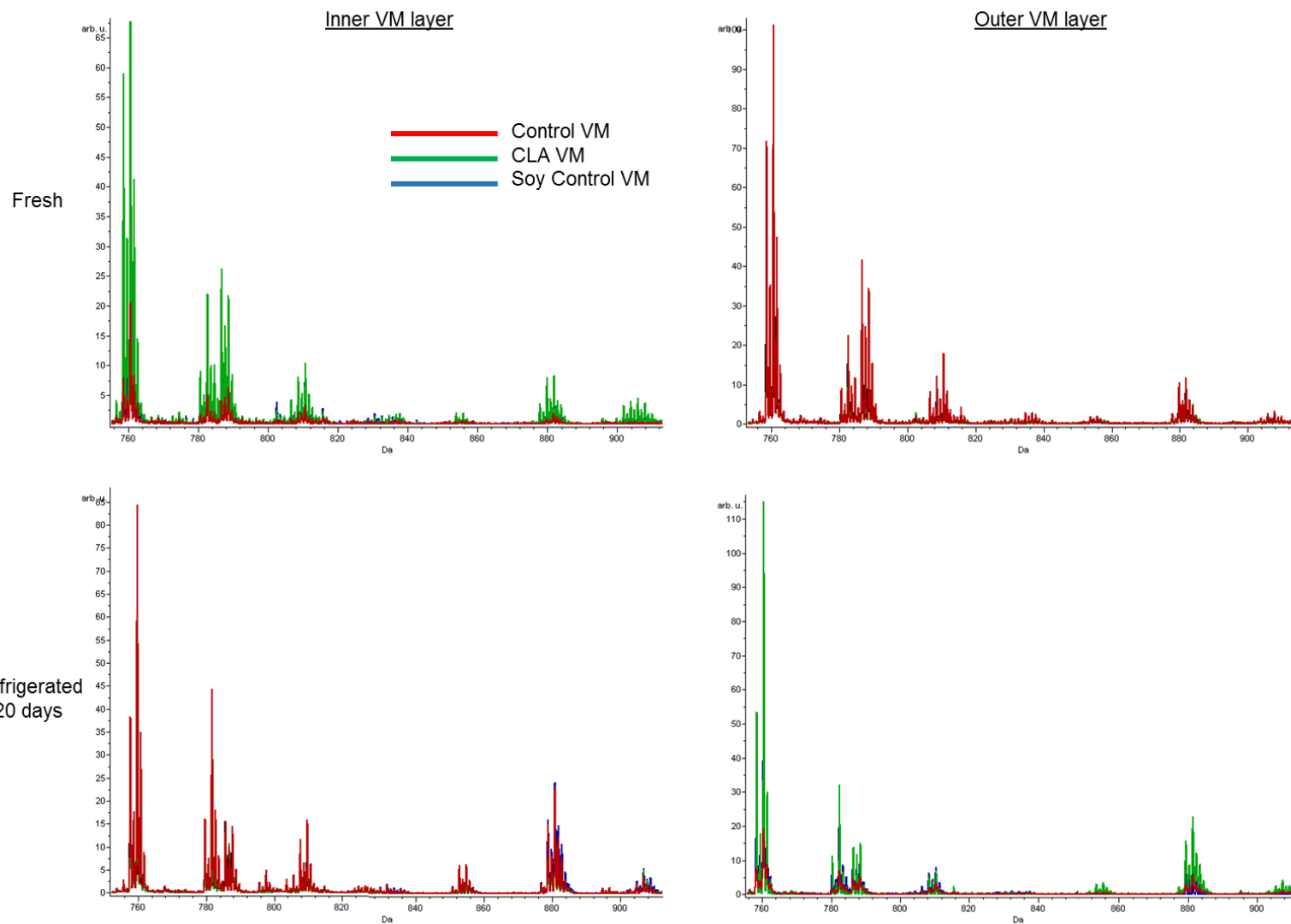
Fatty acyl residue (mol %)	Control VM	CLA VM	Soy control VM
16:0	34.3 \pm 0.6a	35.2 \pm 0.6a	31.9 \pm 0.7b
16:1	2.6 \pm 0.2a	1.1 \pm 0.1b	1.5 \pm 0.1b
18:0	9.0 \pm 0.4b	13.2 \pm 0.2a	9.4 \pm 0.2b
18:1	41.4 \pm 0.7a	30.7 \pm 0.06c	36.2 \pm 0.08b
18:2	10.8 \pm 0.76c	15.8 \pm 0.9b	19.1 \pm 0.6a
18:3	1.6 \pm 0.4a	1.4 \pm 0.5a	1.3 \pm 0.2a
<i>cis</i> -9, <i>trans</i> -11 CLA	0 \pm 0b	0.15 \pm 0.03a	0 \pm 0b
<i>trans</i> -9, <i>cis</i> -11 & <i>cis</i> -10, <i>trans</i> -12 CLA	0 \pm 0b	0.17 \pm 0.02a	0 \pm 0b
<i>trans</i> -10, <i>cis</i> -12 CLA	0 \pm 0b	0.25 \pm 0.02a	0 \pm 0b
<i>trans</i> -9, <i>trans</i> -11 and <i>trans</i> -10, <i>trans</i> -12 CLA	0 \pm 0b	0.72 \pm 0.04a	0 \pm 0b
20:4n-6	0.1 \pm 0.02b	1.1 \pm 0.1a	0.1 \pm 0.02b
22:6n-3	0.2 \pm 0.06b	0.3 \pm 0.05ab	0.4 \pm 0.05a

Table 4. Chicken egg vitelline membrane phosphatidylcholine (PtdCho) and triacylglycerol (TAG) species detected on the inner and outer layers of the VM by Direct MALDI-TOF MS. The treatment groups are standard control eggs, CLA-enriched soy oil eggs, and control soy oil eggs. This analysis was performed in triplicate on six membranes from each treatment egg group on both fresh and refrigerated VM. P-values < 0.05 letters indicate significant differences among the three treatment groups.

	Fresh Vitelline Membranes					Refrigerated Vitelline Membrane												
	Inner VM			Outer VM		Inner VM			Outer VM									
	CLA	Soy Control	Soy control	Pr>F	CLA	Soy Control	Soy control	Pr>F	CLA	Soy Control	Soy control	Pr>F						
PtdChom/z						PtdCho												
34:1	760.6	77.9	34.1	65.9	0.004*	36.3	41.1	46.7	0.06	34:1	80.9	82.1	80.3	0.52	36.6	42.1	40.7	0.09
34:2	758.6	32.9	5.4	18.8	0.003*	2.1	3.5	3.1	0.31	34:2	37.4	36.9	37.2	0.43	37.5	9.8	32.4	0.007*
36:0	790.6	4.5	3.8	5.1	0.47	5.2	4.8	5.2	0.13	36:0	10.2	9.7	9.9	0.36	12.9	6.1	15.4	0.04*
36:1	788.6	15.4	11.9	12.8	0.06	16.3	15.4	16.6	0.20	36:1	15.2	16.7	14.9	0.21	8	3.5	8	0.03*
36:2	786.6	22.5	6.2	16.8	0.002*	5.0	6.9	6.8	0.18	36:2	16.3	16.0	15.9	0.34	6.1	3.8	8.2	0.01*
36:4	782.6	16.0	4.5	16.8	0.002*	11.3	8.9	9.5	0.78	36:4	17.1	16.8	17.0	0.41	25	11.9	26.4	0.01*
38:4	810.6	8.2	3.5	7.2	0.003*	7.4	8.9	9.2	0.14	38:4	15.3	15.5	14.9	0.48	9.2a	5.0b	10.1a	0.007*
38:6	806.6	8	3.5	7.5	0.006*	2.4	2.9	2.7	0.49	38:6	9.7	10.2	9.9	0.35	6.5	5.8	7.1	0.06
TAG						TAG												
50:0	852.8	4.8	4.2	3.8	0.14	4.9	4.2	4.4	0.16	50:0	3.3	6.3	6.1	0.001*	4.9	3.3	4.3	0.13
52:0	880.8	2.6	2.2	2.7	0.10	2.7	2.3	2.6	0.10	52:0	19.8	23.5	25.9	0.01*	27.3	7.3	15.2	0.006*
52:1	878.8	7.7	3.6	4.3	0.04*	3.2	1.9	2.6	0.10	52:1	3.1	1.6	2.6	0.20	15.0	13.1	14.2	0.21
52:2	876.8	3.1	2.5	3.0	0.09	3.0	2.5	3.1	0.09	52:2	14.6	16.8	17.2	0.02*	8.1	5.0	6.2	0.10
54:2	904.8	4.5	1.8	2.6	0.03*	4.2	3.5	3.8	0.14	54:2	3.3b	4.0b	7.6a	0.02*	4.1	2.5	2.8	0.24
54:0	908.9	5.7	2.6	3.5	0.02*	4.5	2.5	2.8	0.08	54:0	3.5b	3.7b	8.6a	0.006*	4.5	2.5	2.8	0.08

* Indicate significant differences at 0.05 α -level.

Figure 3. Lipid profiles of the inner and outer layers of the chicken yolk vitelline membrane spectra analyzed direct MALDI-TOF MS. Three different VM types were compared, including those from: Control yolks, CLA-rich yolks, and Soy control yolks.



CHAPTER 7. Characterization of chicken yolk vitelline membrane proteins using eggs enriched with conjugated linoleic acid

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Abstract

The chicken egg vitelline membrane (VM) separates the yolk from albumen. The objective of this study was to determine protein differences in VM of conjugated linoleic acid (CLA) rich eggs, relative to control egg VM. Recently we characterized the lipids of VM using direct MALDI TOF/MS and conductive glass slides, without any prior chemical derivatization. We determined that CLA eggs possessed VM lipids with significantly different abundancies of 9 phosphatidylcholine species and 6 triacylglycerol species. Other significant differences appeared after 20 days of refrigerated storage. This study used the same rapid, direct MALDI technique to compare protein mass spectra of egg yolk VM in CLA-rich, soy control, and standard control eggs, at day 0 and day 20. Fresh VM from CLA-rich yolks had reversed ratios of gallin protein precursors (m/z 4484 and 4597) on the inner membrane layer, relative to both controls. After 20 d of refrigerated storage, gallin protein ratios were similar among treatment groups, but standard control VM contained significantly greater levels of gallin proteins on the inner membrane layer, and significantly lower gallin protein levels on the outer membrane layer. This rapid MALDI analysis allowed collection of lipid and protein mass spectra by simply shifting the mass range during analysis.

Introduction

The vitelline membrane (VM) encloses the chicken egg yolk, separating it from albumen. VM weaken during storage, and dietary lipid modification significantly affects its strength. Recently, our research group published a paper describing the lipid composition of the VM in control and conjugated linoleic acid (CLA)-rich eggs, and how they change during refrigerated storage [1, Chapter 6]. The VM lipids were ionized and detected by direct MALDI-TOF/MS. During data collection, the analyzer simply shifted the mass range and additionally collected protein mass spectra in replication identical to Shinn et al., [1]. This practice was in response to a coinciding study on egg shell membrane that was determining the soluble membrane proteins also by MALDI [2]. The results suggested the presence of several abundant proteins from egg whites, such as ovalbumin, ovotransferrin, and lysozyme as well as many others associated with antimicrobial, biomechanical, cytoskeletal organizational, cell signaling, and enzyme activities. Therefore the objectives of this manuscript were to: describe the VM proteins by rapid direct MALDI and compare to the findings in [2], determine if there are any protein MS differences among CLA-rich and control VM.

Materials and Methods

Dietary Treatments and Egg Production

Refined, bleached, deodorized soybean oil used for CLA-rich soybean oil production and feed preparation was provided by Riceland Foods (Stuttgart, AR). A CLA-rich soybean oil containing 15% CLA in triacylglycerol form was produced by linoleic acid photoisomerization using a pilot scale unit and method of Jain & Proctor [3]. The feed used in this study was a pelleted commercial corn and soybean meal-based broiler finisher diet (Cobb-Vantress, Siloam Springs, AR). Three dietary treatments were used in this study: 1) unmodified commercial feed ("Control"), 2) Control + 10% soy oil ("Soy control") or 3) Control + 10% CLA-rich soy oil ("CLA"). Soy control and CLA diets were prepared by adding 10% (wt.) soy oil to the standard commercial feed and combined using a Hobart stand mixer. The contents of these three diets are presented in Table 1. It should be noted that the commercial diet used was formulated for meat-type chickens, making it markedly deficient in calcium, a critical nutrient for laying hens. Although this was not the ideal feeding regimen for layer chicken eggshell quality, it still allowed determination of

dietary fatty acids on vitelline membrane composition. In this short-term study we did not observe any issues with egg shell quality, although it may have become apparent after a longer feeding duration. Furthermore, by adding 10% of either soy oil or CLA-rich soy oil to commercial feed to create the experimental diets, the nutrient content of the original diet was diluted by 10%.

One hundred single-comb White Leghorn chicks were reared under standard commercial conditions. At 35 weeks, thirty-six hens were randomly assigned to single bird cages in blocks of 6 birds each, separated by 2 empty cages. All hens received the Control diet until the feeding trial began at 36 weeks. Then each diet (Control, Soy control, or CLA) was randomly assigned to 2 blocks of 6 birds each, resulting in 12 birds receiving each treatment diet. Animal procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee (Protocol # 13033).

Hens were fed *ad libitum* for a total of 30 days. After 20 days of treatment feed administration, which allows maximum CLA accumulation in the egg [4], eggs from each treatment group were collected daily for 10 days, and labeled with cage number and date. Eggs collected on days 1-5 of the 10 day collection period were analyzed as “fresh.” These eggs were stored in single layer flats at 4°C for 6 h prior to subsequent analysis. Eggs collected on days 6 - 10 were refrigerated for 20 days at 4°C before subsequent analysis. In summary, duplicate sets of membranes from each hen and storage condition (fresh or refrigerated) were analyzed for fatty acyl residue composition. In addition, triplicate sets (from different collection days) of 6 randomly selected membranes from each treatment group and storage condition were analyzed for lipid composition by direct MALDI-TOF-MS. Due to availability of the MALDI-TOF, only 6 membranes were able to be analyzed from the three selected collection days for fresh and refrigerated eggs.

VM Protein analysis by direct MALDI

The inner and outer surfaces of fresh and refrigerated vitelline membranes from each treatment group were analyzed by direct MALDI-TOF-MS analysis. Six clean, isolated membranes portions (approximately 10x10 mm) were applied to conductive microscope slides (ITO coating, part No. 237001, Bruker

Corporation) with either the inner or outer membrane exposed for MALDI analysis. One molar 2,5-Dihydroxybenzoic acid (DHB) in 90% MeOH, 10% water containing 0.1% trifluoroacetic acid was used as the MALDI matrix. This was applied in 2 μ L aliquots over each membrane, until DHB matrix crystals were visually observable on the membranes and covered the portion completely. After complete drying under vacuum, slides were fitted to a MALDI target slide holder (Bruker Corporation). The slides were imaged by an Ultraflex II MALDI-TOF/TOF with a 200 Hz smart beam laser in the reflector mode. Membrane preparations were screened for their peptide profiles in the mass range of 1–20 kDa. The extraction voltage was set to 25 kV, and matrix suppression was set to $m/z < 200$. Data were acquired using FlexControl (FC 3.0) and FlexImaging 2.0 software under Compass 1.2 settings (Bruker, Billerica, Massachusetts). Twenty spectra from each set of 6 membrane and treatment group were collected. This experiment was repeated in triplicate. The data was processed using Bruker FlexImaging 2.0, Flex Analysis 2.4/3.0, and ClinProTools 2.2 software (Bruker, Billerica, Massachusetts). Proteins were identified in accordance with the reports of chicken egg shell membranes [2].

Statistical analysis: Vitelline membrane FA composition was compared by JMP 11.0 software using 2-way ANOVA to determine the effects of yolk type and storage using Tukey's HSD with and 0.05 alpha level. The lipid intensities were compared in ClinPro Tools 2.2 software to determine significance differences with and 0.05 alpha level.

Results and Discussion

Figure 1 shows the familiar protein mass spectral patterns that were observed in this study and comparable to the published identification in the previous paper on egg shell proteins [2]. MALDI yielded a highly confident sequence tag (YCSNTCSKTQI) based on observed c ions (N-terminal protected) from m/z 4597. MASCOT sequence query and MS/MS search using MALDI-LIFT-TOF/TOF data and blast search against the NCBI Gallus database all resulted in significant hits against the protein precursor named "gallin' with a sequence LVLKYCPKIGYCSNTCSKTQIWATSHGCKMYCCLPASWKW, matching m/z 4597. Almost the same sequence, but without the N-terminal leucine (L), is a perfect match to m/z 4484. The peak at m/z 4778, although, showed three disulfide bonds from MALDI-TOF-MS results. Its

identification was not possible through the initial means, most likely due to insufficient amounts of material. However, the LC-MS/MS data from the methanol-extracted proteins identified this peptide to be gallinacin 10.

In fresh inner VM both control type membranes had similar 4484 to 4597 ratios, with 4597 as more intense than 4484. The intensity of both of these proteins was somewhat significantly greater in standard control eggs ($p = 0.06$). However, the incorporation of CLA essentially switched the ratio for 4484:4597, with the former having a greater intensity than the latter. In the outer membrane of fresh VM, all three egg types showed similar ratios of 4484:4597 with the former protein having a greater intensity than the latter. However, These protein MS intensities were significantly greater in soy control VM, relative to both standard control and CLA-rich VM.

In the refrigerated VM on the inner layer the intensities of 4484 and 4597 were significantly greater in standard control VM, relative to both enriched egg types ($p < 0.001$). Additionally, the m/z 4778, which corresponds to gallinacin 10, was not apparent in fresh membranes, but becomes so in analysis of the aged membranes. There were no differences in the abundance of gallinacin 10 on either the inner or outer membrane membrane layer. On the outer aged membrane layer, 4484 and 4597 were significantly greater in both the soy control and CLA-rich VM, relative to the standard control ($p < 0.001$).

This experiment was repeated in triplicate and showed consistent results with the previously published paper [2] as well as changes in mass spectra during storage. The major peptides identified in the direct analysis of egg yolk vitelline membrane were 3 defensin-like peptides corresponding to m/z 4484, 339 4597, and 4778 all of which showed to contain 3 disulfide bonds common to most avian beta defensins. Two of these peptides, m/z 4484 and 4597, were of interest because both were identified as gallins with the former being shorter by a single N-terminal amino acid leucine. This experiment utilized the identification of these proteins in egg shell membranes [2] and the rapidity of MALDI to quickly determine distinct differences in vitelline membrane proteins as a result of CLA incorporation and storage, and both of these variables have not been described in VM literature.

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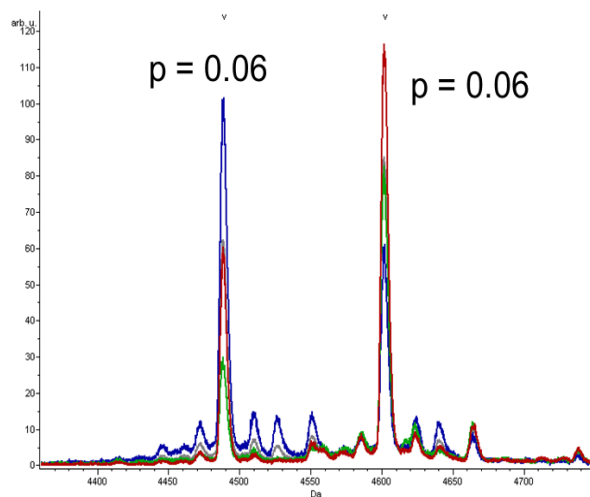
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Tables and Figures

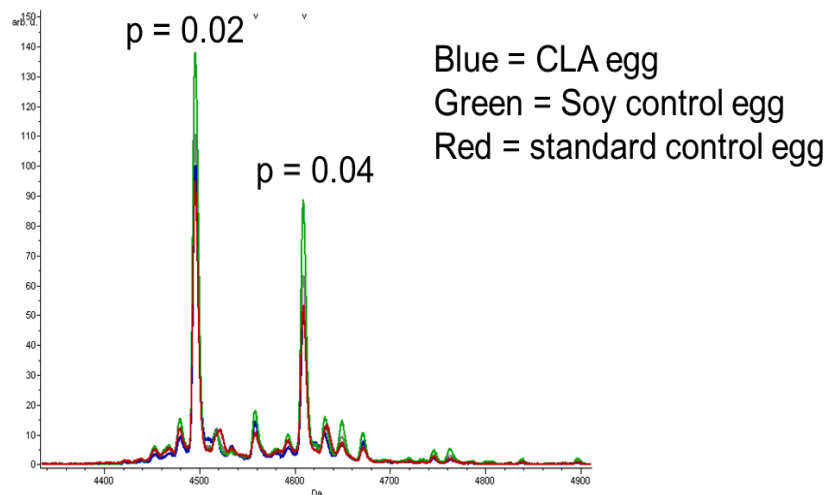
Figure 1. Vitelline membrane protein spectra for fresh and refrigerated CLA-rich or control eggs: Gallin protein precursor (m/z 4484, 4597), and gallinacin 10 (m/z 4778).

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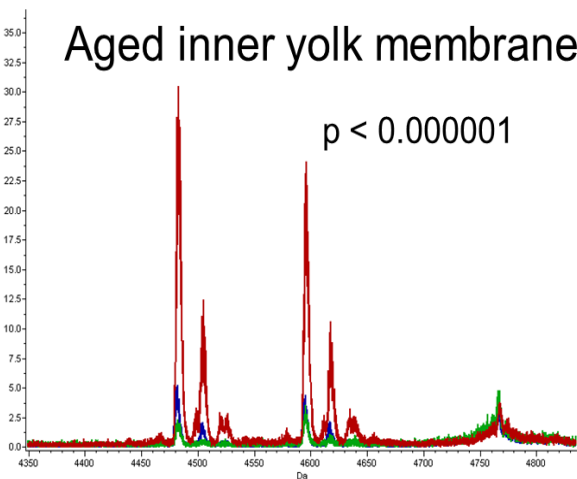
Fresh inner yolk membrane



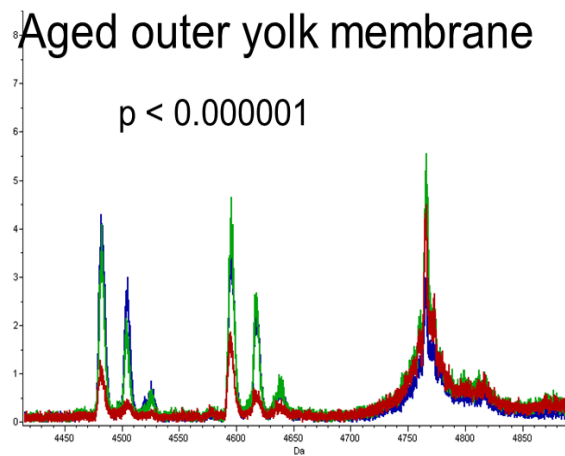
Fresh outer yolk membrane



Aged inner yolk membrane



Aged outer yolk membrane



CHAPTER 8. Effect of feeding CLA on plasma and granules fatty acid composition of eggs and prepared mayonnaise quality

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Abstract

Eggs rich in *trans, trans* conjugated linoleic acid (CLA) are significantly more viscous, have more phospholipids containing linoleic acid (LA), and more saturated triacylglycerol species than control eggs.

However, the fatty acid (FA) composition of yolk plasma and granule fractions are unreported.

Furthermore, there are no reports of mayonnaise rheological properties or emulsion stability by using

CLA-rich eggs. Therefore, the objectives were 1) compare the FA composition of CLA-rich yolk granules

and plasma, relative to standard control and LA-rich control yolks 2) compare the rheological properties of

mayonnaise prepared with CLA-rich eggs to control eggs and 3) compare the emulsion stability of CLA-

yolk mayonnaise. CLA-rich eggs and soy control eggs were produced by adding 10% CLA-rich soy oil or

10% of control unmodified soy oil to the hen's diet. The eggs were used in subsequent mayonnaise

preparation. CLA-yolk mayonnaise was more viscous, had greater storage modulus, resisted thinning,

and was a more stable emulsion, relative to mayonnaise prepared with control yolks or soy control yolks.

Introduction

Conjugated linoleic acid (CLA) is an 18-carbon fatty acid, with various positional and geometric isomers, and is found mostly in dairy and beef products (Whigham *et al.*, 2000). CLA has been shown to possess many positive human health effects, including anti-carcinogenic properties (Cesano *et al.*, 1998, Kim *et al.*, 2002), the capacity to combat obesity and atherosclerosis (Feitoza *et al.*, 2009, Nicolosi *et al.* 1997), decrease the risk of diabetes (McGuire & McGuire, 1999), and improve immune function (Bassaganya-Riera *et al.*, 2012).

Approximately 3.2 grams of CLA need to be consumed daily to realize the health benefits (Bervens *et al.*, 2000, Mougios *et al.*, 2001). However, consumption of ruminant CLA sources are limited because they are also high in saturated fat and cholesterol (Mcguire, 1999, Mougios *et al.*, 2001). Jain and Proctor (2008) developed a process to produce a 20% CLA-rich soy oil by photoisomerization of soybean oil linoleic acid in the presence of an iodine catalyst. *Trans, trans (t,t)* CLA was the predominant CLA geometrical isomer. Gilbert *et al.* (2011) demonstrated that when obese Zucker rats consumed this CLA-rich soy oil, their total serum cholesterol and LDL cholesterol were reduced by 41% and 50%, relative to rats fed a soy oil control diet. Also their liver lipid contents and weights were reduced by 35% after 30 days. Recently, *t,t* CLA has shown greater anti-carcinogenic and anti-atherogenic effects in mouse and rat models, in comparison to *cis,trans* isomers (Islam *et al.*, 2010, Shah *et al.*, 2013).

The CLA-rich soybean oil was subsequently fed to chickens as 1.5% of their diet to produce CLA-rich egg yolks (Shinn *et al.* 2015a). Maximum CLA yolk accumulation occurred after 12 days of feeding, with *t,t* CLA isomers being the most abundant, followed by *trans*-10, *cis*-12 CLA.

CLA-rich and control yolks were also produced to determine the fat content and intact triacylglycerol and phospholipid species present (Shinn *et al.*, 2014). Extractions resulted in a total lipid content of $34.7 \pm 0.05\%$ per volume of yolk with no significant difference in egg type.

A later study on the effects of CLA-rich soy oil on hen egg quality (Shinn *et al.* 2015b) showed that eggs obtained with the CLA-rich soy oil diet had similar yolk size to the non-CLA controls and saturated fat was increased by only 28%. Furthermore, fresh CLA-yolks were significantly more viscous than fresh control yolks, and CLA- rich yolks maintained viscosity for 20 d, while control yolk viscosity significantly decreased (Shinn *et al.*, 2015b). CLA-rich yolk viscosity had not been previously reported. These findings

were in contrast to previous CLA egg enrichment studies with *cis*, *trans* CLA mixtures which produced smaller yolks and up to a 34% increase in yolk saturated fatty acids (Aydin *et al.*, 2001).

The yolk comprises of granules, consisting of 10% lipids, suspended in a liquid plasma, containing 90% lipids (Belitz & Grosch, 1986). Low-density lipoprotein (LDL) is present in both the granule and plasma yolk fractions and is regarded as responsible for yolk's emulsifying properties (Mine & Bergougnoux, 1999). The plasma contains predominantly triacylglycerols, while the granules contain the majority of phospholipid species (Anton and Gandemer, 1997). Mayonnaise is an oil-in-water emulsion containing at least 65% oil and egg yolk (U.S. FDA) and the yolk provides the emulsifiers to stabilize the emulsion. The "film" around the emulsified oil droplets in mayonnaise is composed of yolk plasma-LDL (Chang *et al.*, 1972, Ford, 2004). The plasma film and yolk granule particles serve as "bridges" between the oil droplets in an emulsion, and this granule-oil droplet network holds the formula in a gelatinized structure, without lipid droplets coalescing (Chang *et al.*, 1972, Langton *et al.*, 1999). Both the plasma and granule fractions play an integral role in mayonnaise emulsion stability and undoubtedly influence its rheological properties (Ford *et al.*, 2004). However, the fatty acid compositions of these two yolk fractions is unreported. Therefore, a comparative study of the fatty acid composition of CLA-rich yolk plasma and granule fractions with those of control yolk fractions may provide further insight on how yolk fatty acid profile affects mayonnaise quality.

Furthermore, since CLA in yolks increases yolk viscosity (Shinn, 2015b), modifying the egg yolk fatty acid profile with CLA in yolks may also affect the rheological properties mayonnaise and emulsion stability. Mayonnaise emulsion viscosity is an important quality attribute (Weenen *et al.*, 2003), and it is thought that the continuous phase viscosity controls mayonnaise stability and texture (Langton, 1999). Modifying the egg yolk lipid profile in the continuous aqueous phase of the emulsion may have significant effects on mayonnaise quality. However, there are no rheological studies reporting the effect of CLA-rich yolks on mayonnaise quality. Mayonnaise quality includes mayonnaise gelatinization, flow, and spread-ability, and these properties can be determined instrumentally by assessing oscillatory stress and storage modulus (G'), creep and recovery curves, and viscosity, respectively (Whittingstall). Mayonnaise oscillatory stress and resulting elasticity measurements (G') indicate the degree of "gel" structure and the thickness that will be encountered when the product is in a container (Whittingstall). Mayonnaise creep and recovery

behavior describes how the mayonnaise responds to low steady stresses, like gravity after being spooned from a vessel (Whittingstall, 2014). Relating viscosity to shear rate results in simple flow curves that indicate mayonnaise spreadability (Whittingstall).

Additionally, emulsion stability is also an important quality attribute that indicates mayonnaise shelf-life before possible phase separation. By considering how CLA incorporation affects fatty acid composition and subsequent mayonnaise rheology and stability we may gain a better understanding of egg yolk emulsion properties and capabilities, and how these are modified by yolk lipid alterations.

Therefore, the objectives of this study are 1) Compare the fatty acid composition of CLA-rich yolk granules and plasma with that of yolk granules and plasma of less viscous control yolks 2) Compare the rheological properties of mayonnaise prepared with CLA-rich eggs, relative to controls 3) Compare the emulsion stability of CLA-egg mayonnaise, relative to controls.

Experimental Section

Materials

The refined, bleached, deodorized soybean oil used for CLA-rich soybean oil production, feed preparation, and mayonnaise formulation was provided by Riceland Foods (Stuttgart, AR). Other mayonnaise ingredients were purchased from a local supermarket. All lipid extraction solvents were analytical grade. The fatty acid methyl ester (FAME) internal standard used were purchased from Supelco (Bellefonte, PA).

Methods

Feed Preparation and egg collection

CLA-Rich Soy Oil Production: A 15% CLA-rich soybean oil was produced by linoleic acid photoisomerization using a pilot scale unit and method of Jain & Proctor (2008). CLA-rich soy oil was analyzed for fatty acid composition and contained: 15% CLA, 12.2% C16:0, 0.09% C16:1(n-7), 4.2% C18:0, 25.9% C18:1(n-9), 38.5% C18:2(n-6), and 3.6% C18:3(n-3).

Feed Preparation: The 15% CLA soybean oil was combined with pelleted commercial corn and soybean meal-based finisher diet (Cobb-Vantress, Siloam Springs, AR). The commercial pelleted diet contained

2761 kcal/kg metabolizable energy and 15.43% crude protein. Feed was formulated without meat or animal by-products to meet or exceed minimum National Research Council standards (1994) for all ingredients. The CLA diet was produced by adding 10% (wt.) CLA-rich soy oil to the standard commercial feed and combined using a Hobart stand mixer. A soy oil control diet was similarly prepared, and the unmodified feed was the standard control diet.

Diet administration: One hundred single-comb white leghorn chicks were reared under standard commercial conditions. At 35 weeks, thirty-six hens were randomly chosen and assigned to single bird cages in blocks of 6 birds each, separated by 2 empty cages. All hens received the same standard commercial finisher diet until the feeding trial began at 36 weeks. Each soy oil diet (15% CLA soy oil and conventional soy oil) was randomly assigned to 2 blocks of 6 birds each, resulting in 12 birds receiving each treatment diet. The remaining twelve hens continued to receive standard commercial feed without additional soy oil and served as a standard control group. Animal procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee (Protocol # 13033).

Egg Collection: After 12 days of treatment feed administration, eggs were collected daily for 10 days, counted, and labeled with cage number and date. Eggs were stored in a walk in cooler at 4°C for 24 h prior to subsequent analysis.

Lipid extraction and fatty acid analysis of yolk plasma and granules

Plasma and granule fractionation: Yolks were separated from albumen and rolled on a *Kimwipe* to remove any residual adhering albumen. Yolks from the duplicate treatment blocks were pooled (6 eggs = 1 sample replicate). Duplicate 20 g yolk samples from all three egg types were combined with 1 mL of 1% NaCl in deionized water and centrifuged at 10,000 rpm for 1 h (Belitz & Grosch, 1986). The top plasma layer was decanted from the bottom granule layer. Both fractions were centrifuged again at 10,000 rpm for 1 h to remove any contaminating fraction.

Plasma and granule lipids extraction: Plasma and granule total lipids were extracted from duplicate samples from each egg type using a rapid hexane/isopropanol method (Shinn and Proctor, 2013). A 0.3

g plasma sample or 2 g granule sample was collected in a 7 mL glass vial and dissolved in 5 mL hexane/isopropanol (1:1, v/v), vortexed for 5 min, and allowed to settle. The liquid containing the lipid extract was decanted into a 50 mL centrifuge tube and used for subsequent FAME preparation. In addition, the total lipids in the plasma and granule fractions were quantified using the above-mentioned extraction method and subsequent extract evaporation and gravimetric quantification.

Fatty acid analysis: Duplicate lipid samples were derived to fatty acid methyl esters (FAMES) using a rapid, micro FAMES preparation method modified for the volume of lipid extract needed from each fraction (Lall et al., 2008). Decanted extraction samples in 50 mL centrifuge tubes were combined with 0.5 mL of a prepared 1% (wt.) heptadecanoic acid (C17:0) methyl ester solution in hexane. Then 1 mL of toluene and 4 mL of 0.5 M sodium methoxide in methanol were added to each tube. The centrifuge tubes were placed in a 50°C water bath for 10 min with constant agitation and then removed and cooled at ambient temperature for 2 min. Glacial acetic acid, 0.2 mL, was added to each tube to inhibit sodium hydroxide formation. Five mL of distilled water was added, followed by 2 mL of hexane. The tubes were vortexed for 2 min and then the immiscible layers were allowed to separate. The upper layer was pipetted out and dried over anhydrous sodium sulfate for 15-20 s.

Duplicate fatty acid profiles were obtained for each FAME preparation by GC with a SP 2560 fused silica capillary column (100m x 0.25 mm i.d. x 2 µm film thickness; Supelco Inc. Bellefonte, PA), and a flame ionization detector (FID model 3800, Varian, Walnut Creek, CA). Samples of 3.0 µL were injected by autosampler. The FID settings were as follows: oven temp = 250°C; sensitivity = 12, He gas = 30 mL/min, H₂ = 31 mL/min, air = 296 mL/min, and over program time = 111 min.

Total fatty acid composition, CLA concentration and CLA isomer concentration were calculated by the following equation:

$$[\% \text{ fatty acid}] = ([\text{HME}] \times \text{sample peak area} \times \text{relative response factor}) / \text{HME peak area}$$

where HME is the heptadecanoic (C17:0) methyl ester internal standard.

The position of double bonds in CLA-rich soybean oil produced by photoisomerization (Jain & Proctor, 2006) were previously determined by silver ion HPLC (Shah *et al.*, 2012). Fatty acid compositions were presented as % FA of either the plasma or granule lipid content.

Mayonnaise Preparation

Low egg and high egg mayonnaise formulations, were prepared in duplicate using either CLA, soy control, or control egg yolks. Standards of identity for mayonnaise require at least 65% oil in the formulation (U.S. FDA, 2014). Therefore, the low egg recipe contained 16% egg yolk: 73% soy oil (wt:wt), and the high egg recipe contained 24% egg yolk: 65% soy oil (wt:wt) (Jones, 2007). Vinegar and salt percentages were 10% and 1%, respectively, in both formulations. Mayonnaises were prepared using a KitchenAid® stand mixer (KitchenAid, St. Joseph, MI) fitted with a whisk attachment (Jones, 2007). Vinegar and salt were mixed on speed 10 for 1 min. Egg yolk was then incorporated at speed 6 for 2 min. Speed was increased to 10 and oil was added steadily over a 4 min period. The mixer was turned off and a spatula was used to move all mayonnaise down off the sides of the mixing bowl. Mayonnaise was finally mixed for 2 min at speed 10. Mayonnaise from each duplicate set were refrigerated at 5°C in 50 mL centrifuge tubes for 24 h before subsequent analyses.

Mayonnaise Imaging

Confocal laser scanning microscopy (CLSM) was used to study the mayonnaise microstructure. The duplicate mayonnaise preparations were diluted 3x with deionized water in triplicate, vortexed, and then applied to microscope slides. A Leica SP5 confocal microscope (Leica Microsystems Inc., Buffalo Grove, IL) was used to view the samples. Laser configurations were as follows (visible range): 458 nm set at 19%, 476 nm set at 18%, and 488 nm set at 19%. The hybrid detector's spectral range was set at 498-581 nm.

Mayonnaise rheology

All rheological measurements were carried out with and AR-2000 Rheometer (TA Instruments, Delaware, US) fitted with a 40 mm sandblasted geometry and 1000 µm gap. Each prepared duplicate sample was analyzed in duplicate for each of the rheological measurements. Samples were dispensed on the lower plate using a 5000 µL autopipette set to 1650 µL.

Oscillatory stress: A Rheometer program was used with a conditioning step of 5 min at 5°C, stress sweep from 1 – 160 Pa, and a constant 1 Hz frequency. Storage modulus G' and loss modulus G'' were then determined.

Creep and Recovery: Mayonnaise creep and recovery behavior describes how the mayonnaise responds to stress application and removal and correspond to spread-ability. These were determined with a constant applied stress of 15 Pa for 180 s, followed by a recovery step at 0 Pa for 360 s.

Viscosity: Viscosity was determined by measuring simple flow rates at a shear rate range of 0.001 to 1 s⁻¹ at 5°C in duplicate from all mayonnaise preparations.

Mayonnaises emulsion stability

Emulsion stability was determined by centrifugation to quantify the amount of oil separation that occurs (Nielsen, 2010). Duplicate 10 g mayonnaise samples from each preparation were weighed into 50 mL centrifuge tubes and centrifuged for 6 h at 10,000 rpm.

Statistical analysis

All statistical analyses were performed using JMP 11 (SAS Institute, Inc., Cary, NC) statistical software. Oscillatory stress and viscosity were analyzed by comparing the overall means by one-way ANOVA using a student's t-test with an α -level of 0.05. Emulsion stability was compared using 2-way ANOVA to determine the effect of egg type, oil content, and their interaction using a student's t-test with an α -level of 0.05. Fatty acid composition was compared by one-way ANOVA using a student's t-test with an α -level of significance at 0.05.

Results and Discussion

Fatty acid analysis of yolk plasma and granules

Table 1 shows the plasma and granule fatty acid (FA) composition of eggs produced on the standard control diet, and this diet supplemented with either control conventional soy oil or CLA-rich soy oil. The

granules contained 10% lipid, and the plasma contained 90% lipid, regardless of yolk type. Total CLA was 0.12% of total fatty acids in the granules, and 1.1% of total plasma FA.

Plasma: The plasma fraction contains the majority of the lipid present in the yolk. CLA plasma had significantly greater saturated fatty acid (SFA) than soy control yolk plasma, but was not significantly different from control yolk plasma. CLA-yolk plasma monounsaturated fatty acid (MUFA) concentration was significantly lower than soy control yolks, but significantly different control yolk plasma MUFA. CLA polyunsaturated fatty acids (PUFA) concentration was only slightly lower than soy control plasma, but the differences among yolk types was insignificant. In addition, CLA plasma contained the intermediate long-chain PUFA concentration.

Granules: Granules contain only 10% lipid, which is considerably less than plasma with 90% lipid. Control yolk contained the highest granule SFA levels, and was significantly greater than both CLA and soy control granule SFA. Soy yolk granules, contained significantly greater MUFA concentration relative to CLA yolk granules, and control yolk granules contained the lowest MUFA concentration. PUFA concentration in granules were not significantly different among yolk types.

The increase in SFA, and subsequent decrease in MUFA and long-chain PUFA in *trans, trans* CLA oil-rich yolks relative to soy oil-rich yolks has been well reported (Shinn, 2015a & b,) and plasma FA profiles were shown to be similarly altered. The significant increase in SFA and inclusion of CLA in yolk plasma had significant effects on mayonnaise microstructure, rheology, and emulsion stability, and will be further discussed in the context of the following data.

Mayonnaise Imaging

Figure 1 shows CLSM micrographs of all prepared mayonnaise samples. The first column of images shows the high-egg mayonnaise formulation containing 24% yolk and 65% oil. Overall, these samples show a network of oil droplets suspended in a much greater volume of yolk granules. In contrast, the low-egg formulation (16% yolk, 73% soy oil) shows an oil droplet network positioned in a less compact yolk granule environment. It has been previously shown by CLSM that mayonnaise prepared with >70% vegetable oil generally has less granule density and connecting network between the droplets, and the

granules are more difficult to visualize than mayonnaise prepared with less oil (Langton *et al.*, 1999), which we confirmed in this study.

In the high-egg formula, the micrograph of mayonnaise made with CLA-rich yolks appears similar to the control mayonnaise micrograph in that they possess more homogeneous oil droplet sizes. Alternatively, the soy yolk mayonnaise has a few much larger oil droplets dispersed throughout the continuous phase. This difference was apparent in triplicate slide preparations, so we can assume that the large oil droplets present in the soy oil formulas are not preparation artifacts. In addition, the oil droplet does not seem to be as well defined in soy control mayonnaise samples as they are in CLA and control mayonnaise samples.

In the low-egg formula, the CLA low-yolk mayonnaise still retains easily defined oil droplets that seem slightly larger than those in the CLA high-yolk mayonnaise. However, the interface of the oil droplets in the control mayonnaise are ill-defined in the micrograph, resulting in an oil phase that seems much more voluminous, relative to the continuous phase. Furthermore in the soy control mayonnaise the interface between the oil droplets and the continuous phase is even more difficult to distinguish in the micrographs. In addition, there were large yolk droplets that appeared to be enclosed in larger oil droplets, which were then surrounded by more continuous aqueous phase. These enclosed yolk droplets were apparent in triplicate slide preparations.

Processing conditions such as the mayonnaise oil content and the speed of homogenization have been reported to significantly affect the microstructure of mayonnaise (Langton *et al.*, 1999). Greater oil concentrations result in oil droplets that are much more tightly packed together, resulting in oil droplet deformation (Langton *et al.*, 1999). Higher homogenization speeds result in much smaller oil droplets that are more evenly distributed throughout the emulsion (Langton *et al.*, 1999) This is the first report of the effect CLA-rich yolks, or any egg with an altered lipid composition on mayonnaise microstructure. The inclusion of CLA increased SFA, decreased MUFA, and resulted in smaller and more evenly dispersed oil droplets, relative to control yolk mayonnaises.

Mayonnaise rheology

3.3.1 *Oscillatory stress*: Figure 2 shows the elastic response (G') to oscillatory stress of the two mayonnaise formulations prepared with either CLA-rich, soy control, or control yolks. The low-egg (16% yolk, 73% soy oil) formulas prepared with CLA and control exhibited similar elastic responses, while soy egg mayonnaise had a significantly lower elastic response ($p < 0.0001$). This means that these two samples exhibit similar gel rigidity. The yolk granules have been deemed responsible for the relative stiffness in mayonnaise (Anton, 1999; Chang, 2007), and both CLA and control yolk granules had greater amounts of saturated fat, likely increase the rigidity of the gelled structure. CLA yolk had a smaller proportion of granules, relative to control yolk, but this difference did not weaken the gel formation. In addition, the CLA incorporated in the granules, as well as the relatively lower concentration of MUFA and PUFA, relative to SFA, all likely contribute to CLA mayonnaise having an elasticity response similar to control yolk mayonnaise. The soy control yolk mayonnaise exhibited significantly greater MUFA and PUFA concentrations, relative to both CLA and control yolk mayonnaise. This FA modification seems to have resulted in less rigid gel structure.

However, increasing the yolk content to 24% and decreasing the oil amount to 65% resulted in a CLA-rich mayonnaise that was significantly more elastic than both the soy- and control-egg mayonnaises ($p < 0.0001$). Increasing the yolk content of the mayonnaise did increase the elasticity in all 3 samples, relative to the low egg formulations. However, the CLA mayonnaise had significantly increased gel stability, likely due to a synergistic effect of the increased yolk content in addition to the increased SFA and CLA in the formulation. In addition, the micrographs (Figure 1) showed that CLA-rich mayonnaise with 24% yolk content had smaller and well-defined droplets, relative to the other samples. It has been shown that smaller droplet size results in greater G' (Langton *et al.*, 1999), and our findings confirmed this microstructural and rheological link. However, this effect was not as a result of lower oil content (Langton, 1999), but due to alteration of the yolk FA composition.

Creep and recovery: Figure 3 shows the mayonnaise creep and recovery determinations. Creep tests examine the way a product responds to steady low stresses, elucidating how the mayonnaise would behave under gravity *after* being spooned from a jar. In the high egg formula (24% yolk) CLA mayonnaise and control mayonnaise experience similar % strain levels during creep analysis, and were

significantly greater than soy mayonnaise % strain. The greater % strain measurement indicates that CLA and control mayonnaises had greater compliance to the applied stress. Therefore, these formulations would be more susceptible to gravitational forces after being spooned from a jar in comparison to soy control high yolk mayonnaise, and they would likely spread out more easily in response to gravity, without any additional force on the product.

Decreasing the yolk content increased the creep curves of all three samples (Figure 2B), relative to the high yolk samples (Figure 2A). The decrease in yolk content and subsequent increase in soy oil content resulted in mayonnaise that is much more susceptible to movement under gravity. Generally mayonnaises with greater oil content have lower % strain relative to low-fat mayonnaises (Whittingstall, Langton *et al.*, 1999). However, this study determined that the % strain was greater in all of the 73% oil-containing mayonnaises, relative to the 65% soy oil mayonnaise. In addition, the CLA mayonnaise has significantly lower % strain measurements, relative to control and soy control mayonnaise with the same oil content. This indicates that CLA-mayonnaise with a lower yolk content would appear much more rigid and “rich” after being spooned from the jar, in comparison to the two controls with the same formulation (Whittingstall, 2014).

Viscosity: Figure 4 shows the simple flow curves for the samples relating viscosity to shear rate. Flow behavior relates to mayonnaise spread-ability. In the low egg formulation, control egg and soy egg mayonnaise spread similarly ($p = 0.74$), while CLA mayonnaise would be significantly more resistant to spreading, in regards to both control samples ($p < 0.0001$). However, when yolk content in the mayonnaise formulation is increased, the CLA and soy yolks samples spread similarly, while mayonnaise prepared with control yolks is slightly easier to spread (Figure 2b).

Mayonnaise rheology has been reported in response to different homogenization speeds (Langton *et al.*, 1999) oil concentration and composition (Brandt, 1999) and fat replacers such as gums, starch, and proteins (Clegg, 1999). Increased homogenization speeds can improve mayonnaise gel appearance and reduce spread-ability, as does higher oil content (Langton, Whittingstall), and fat replacers can be used without significant effects on rheological properties (McClements, 2005, Clegg, 1999). However, in

keeping the processing method and mayonnaise formulation constant, this experimental design showed that egg yolk lipids can have a significant effect on egg yolk rheology. More interestingly is that CLA-rich egg yolks increased the mayonnaise's gelled appearance and made it more resistant to slow spreading over time. In addition, yolks produced from feeding chickens non-CLA soy oil resulted in opposite effects.

Mayonnaises emulsion stability

Table 2 shows the relative emulsion stability of mayonnaise preparations. Significantly less oil separated from CLA mayonnaise than the controls in both the high and low egg yolk formulations ($p < 0.0001$), while soy egg mayonnaise had significantly greater separation than both control and CLA mayonnaise. The soy mayonnaise with 65% oil separated even more significantly than soy mayonnaise containing 73% oil. The emulsions stability of a food dressing is a relative concept, given that all dressings are thermodynamically unstable will separate if provided enough time. This experiment showed that modifying the yolks with either control soy oil or CLA-rich soy oil has significant, and opposite effects on emulsion stability.

The yolk granules have been previously attributed as providing the major stabilizing force in mayonnaise because they inhibit the oil droplets from getting too close and coalescing (Ford *et al.*, 2004, McClements, 2005). However, the high yolk formulations exhibited greater oil separation, relative to their lower yolk counterparts. This is despite the fact that the oil droplets appear more separated in all of the high yolk formulations (Figure 1). Therefore, it is likely that other repulsive forces as a result of FA alteration is stabilizing the CLA-mayonnaise more than the controls, and its stabilization is not simply due to the amount of granules present and their particle stabilization mechanism.

Final remarks

The mayonnaise emulsion is stabilized by the surface-active components in eggs including phospholipids, proteins, and LDL (Mine 1998a,b, Anton *et al.*, 2000). These components form an interfacial membrane that is rupture-resistant and produces strong repulsive interactions between oil droplets. While the protein component of the granules has been deemed most responsible for microstructure organization and emulsion stability (Mine, 1999, Ford, 2004, Friberg *et al.*, 2004), this experiment demonstrated that

simply modifying the yolk fatty acid composition can have significant effects on the resulting mayonnaise quality attributes that were considered in this study.

In a previous hen feeding study that used *t,t* CLA in the same dietary concentration as this study (Shinn *et al.*, 2014) CLA-rich yolk phospholipids (PL) had significantly greater lyso-phosphatidyl choline concentrations, and greater concentrations of phosphatidyl choline (PC) 34:2 (16:0/18:2) and 36:2 (18:0/18:2), while the saturated PC species 34:0 (16:0/18:0) was significantly lower in CLA-rich PL. In contrast, the CLA-rich TAG yolk fraction had significantly greater saturated TAG, including: 48:0 (16:0/16:0/16:0), 50:0 (16:0/16:0/18:0), and 54:0 (18:0/18:0/18:0). In addition, CLA-yolk TAG also had significantly lower concentrations of MUFA- and PUFA-containing species. It has been determined that *t*-10, *c*-12 CLA inhibits stearoyl-CoA desaturase, an enzyme that converts C16:0 and C18:0 to C16:1(*n*-7) and C18:1(*n*-9), respectively (Lee *et al.*, 1998, Hur *et al.*, 2007). This leads to more SFA and lower MUFA in egg yolk, and also liver and muscle tissue. While there is no information determining whether *t,t* CLA also inhibits stearoyl-CoA desaturase, *t*-10, *c*-12 CLA was also present in the CLA-rich soy oil at 1% of total FA, and may still be inhibiting the SFA to MUFA conversion. While this experiment did not determine plasma and granule TAG and PL composition, the CLA-rich yolk FA composition modifications and CLA contents are similar to the previous study (Shinn *et al.*, 2014), so it is likely that the TAG and PL compositional changes are synonymous.

In mayonnaise, dispersed oil droplets are surrounded by yolk TAG-rich plasma. The continuous phase consists of dissolved PL-rich granules (Anton, 1999; Chang, 2007). Changing FA, TAG, and PL composition of egg yolk by incorporating CLA, and then using these enriched eggs in mayonnaise preparation increased emulsion stability, reduced oil droplet size, and increased mayonnaise thickness and viscosity, relative to control yolk mayonnaise formulations. CLA-rich mayonnaise not only provides a value-added product, but it is also more viscous, retains its rigid structural appearance longer, and resists over-spreading and thinning, relative to mayonnaise prepared with control yolks.

However, before further development of products enriched in CLA require additional determination of the oxidative stability of CLA-rich eggs and resulting mayonnaise, as well as assessment of sensory

properties. It has been shown that eggs enriched with DHA by adding marine plants to the diet can result in a fishy off-flavor in the egg (Surai & Sparks 2001). Furthermore, eggs enriched with CLA or a combination of CLA and fish oil have shown to be less oxidatively stable, relative to control eggs (Cherain et al., 2007). The oxidative stability of CLA-rich eggs may have unforeseeable effects on the egg's sensory properties or the properties and subsequent shelf-life of food products using CLA-rich eggs.

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Tables and Figures

Table 1. Total fatty acid composition of plasma and granule fractions of egg yolk enriched with CLA, relative to control yolks. Granule fraction contained 10% lipid and plasma contained 90% lipid, regardless of yolk type. FA are presented as % of total fatty acids in either granule or plasma fraction.

Yolk fatty acids	Granules (10% of total FA)			Plasma (90% of total FA)		
	CLA	Control	Soy	CLA	Control	Soy
14:0	0.03	0.04	0.03	0.25	0.24	0.22
16:0	2.57	3.13	2.25	22.93	22.74	20.1
18:0	1.47	1.72	1.05	13.32	11.99	9.64
Total SFA	4.07 ± 0.05b	4.88 ± 0.02a	3.33 ± 0.06c	36.5 ± 0.4a	37.02 ± 0.4a	29.96 ± 0.1b
16:1(n-7)	0.1	0.13	0.16	0.88	1.42	1.37
18:1(n-9)	3.08	2.07	3.69	27.81	33.19	33.18
Total MUFA	3.19 ± 0.1b	2.2 ± 0.01c	3.85 ± 0.03a	28.69 ± 0.6b	29.77 ± 0.5a	34.56 ± 0.6a
18:2(n-6)	2.33	2.63	2.49	21.24	17.94	22.44
18:3(n-3)	0.05	0.05	0.06	0.41	0.29	0.54
20:4(n-6)	0.16	0.16	0.17	1.43	1.53	1.57
20:5(n-3)	0.01	0.02	0.02	0.09	0.15	0.16
22:6(n-3)	0.06	0.06	0.09	0.57	0.51	0.78
Total PUFA	2.62 ± 0.03a	2.92 ± 0.01a	2.82 ± 0.1a	23.74 ± 0.1a	23.01 ± 0.3a	25.49 ± 0.5a
<i>cis</i> -9, <i>trans</i> -11 CLA	0.01	0	0	0.1	0	0
<i>trans</i> -9, <i>cis</i> -11 & <i>cis</i> -10, <i>trans</i> -12 CLA	0.02	0	0	0.17	0	0
<i>trans</i> -10, <i>cis</i> -12 CLA	0.02	0	0	0.18	0	0
<i>cis</i> -11, <i>trans</i> -13 CLA	0	0	0	0.03	0	0
<i>trans</i> , <i>trans</i> CLA	0.07	0	0	0.59	0	0
Total CLA	0.12 ± 0.02a	0b	0b	1.07 ± 0.1a	0b	0b

Figure 1. CLSM micrograph of high-yolk (24% yolk, 65% soy oil) and low-yolk (16% yolk, 73% soy oil) mayonnaise formulas prepared with either CLA-rich, control, or soy control yolks (scale width = 500 nm).

Low egg formula (16% yolk, 73% soy oil)

High egg formula (24% yolk, 65% soy oil)

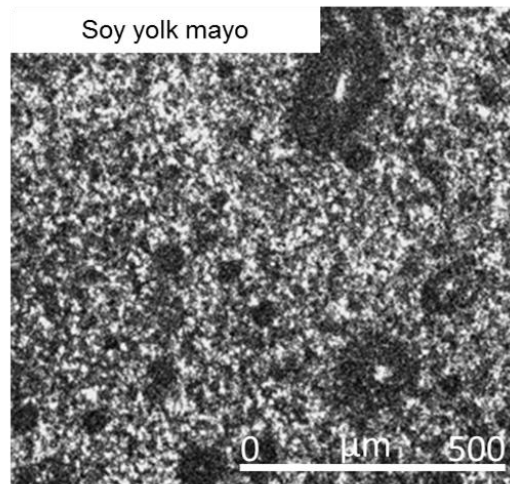
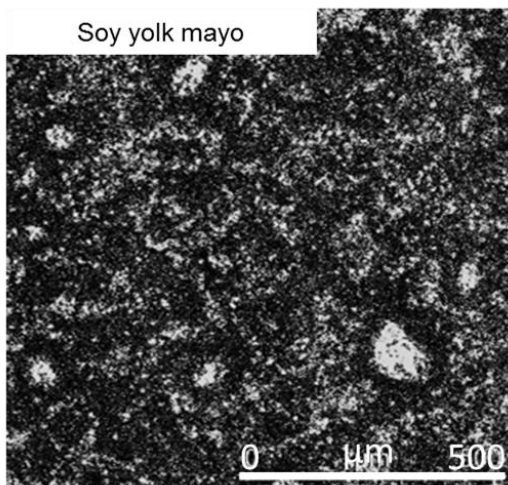
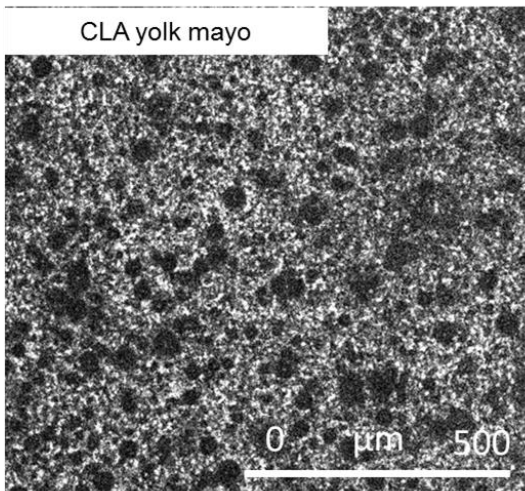
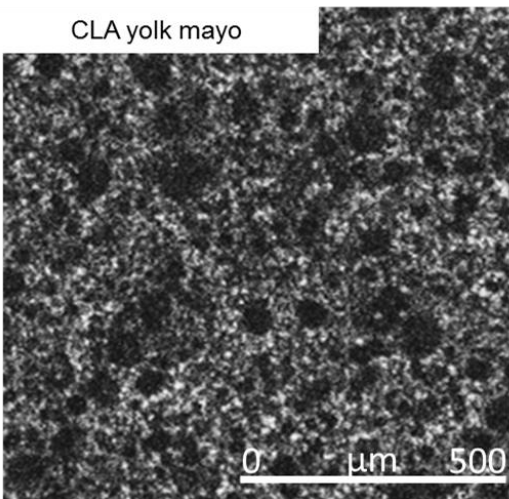
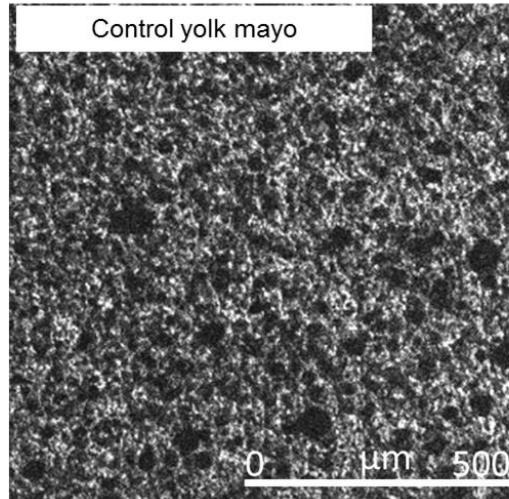
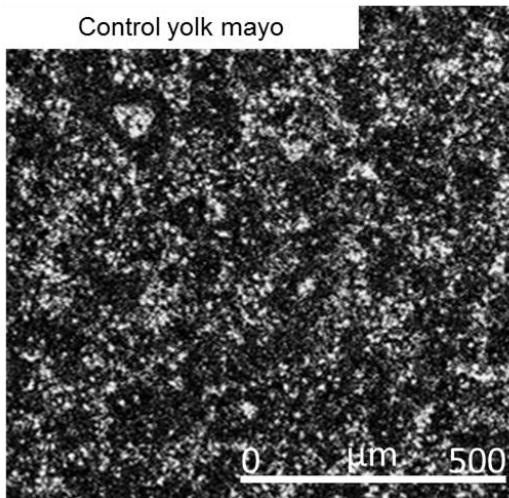


Figure 2. Mayonnaise elastic modulus (G') plotted as a function of oscillatory stress (Pa) for low and high egg formulations prepared with either control yolk, soy control yolk, or CLA-rich yolk. Each analysis was performed in duplicate on duplicate samples and the error bars represent one standard error from the mean.

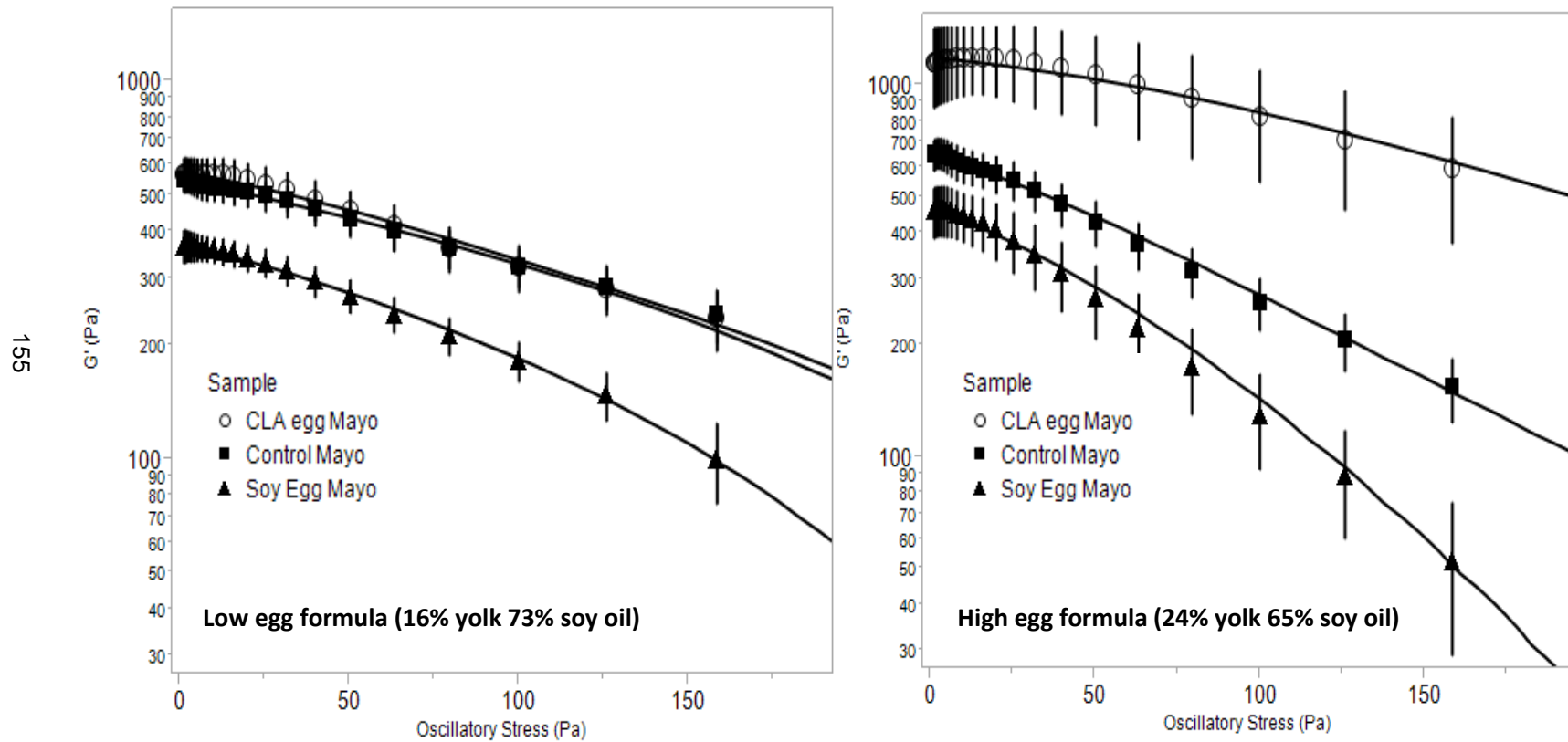


Figure 3. Mayonnaise creep and recovery curves for high and low egg formulations prepared with either control yolk, soy control yolk, or CLA-rich yolk.

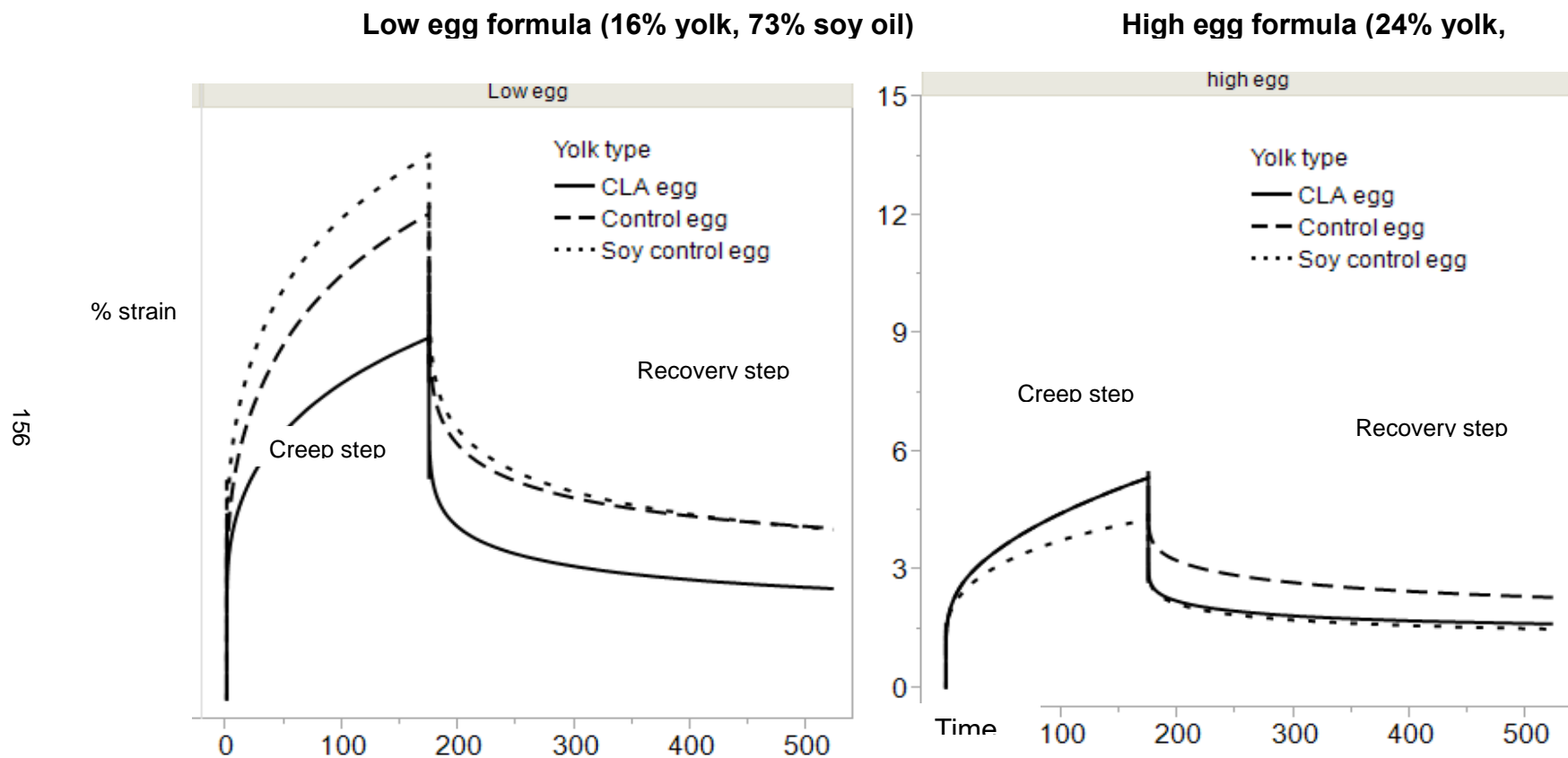


Figure 4. Mayonnaise viscosity (Pa.s) plotted as a function of shear rate (1/s) for low and high egg formulations prepared with either control yolk, soy control yolk, or CLA-rich yolk. Each analysis was performed in duplicate on duplicate samples and the error bars represent one standard error from the mean.

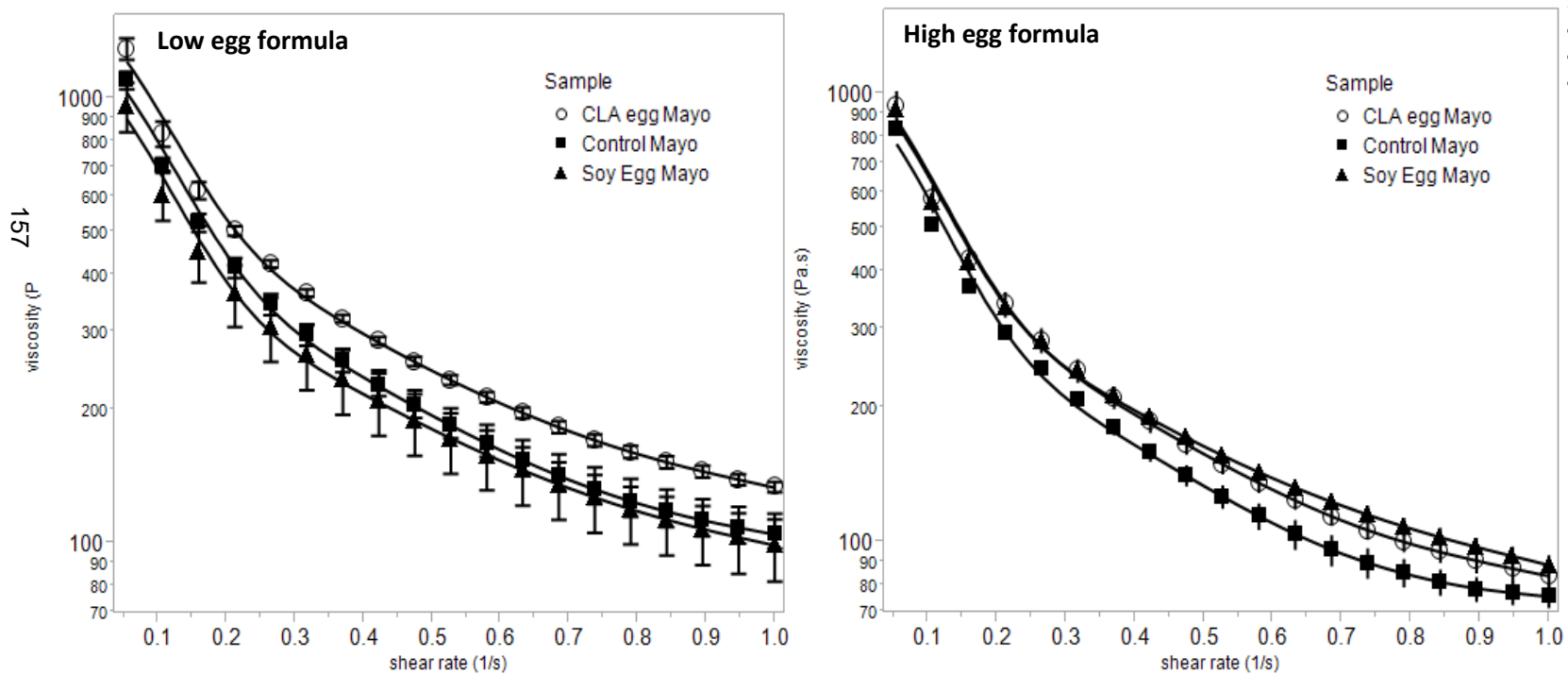


Table 2. Mayonnaise emulsion stability determined by centrifugation at 10,000 rpm for 6 h. The amount of oil that separated after centrifuging was determined in duplicate from duplicate samples. Samples with the same connecting letter are not significantly different at α -level of 0.05.

Sample	Oil separation (%)	
	<i>High egg formula</i>	<i>Low egg formula</i>
CLA Egg Mayo	1.4 ± 0.33d	1.21 ± 0.15d
Control Egg Mayo	3.27 ± 0.45c	2.9 ± 0.24c
Soy Egg Mayo	6.24 ± 0.29a	4.52 ± 0.29b
Effects test	p-value	
Sample	<.0001	
Formulation	0.007	
Sample*Formulation	0.04	

Chapter 9. Conjugated Linoleic Acid (CLA) Rich Eggs: Dried for Convenience and Evaluated for Use in Breakfast Sandwiches

Sara Shinn, Andy Proctor, Nicholas Anthony, Jamie Baum, Jackson Lay Jr.

Introduction

In 2013, 34.6% of adults Arkansans were reported to be obese, with Arkansas ranking as the third most obese state after Mississippi and West Virginia (both 35.1%). Conjugated linoleic acid (CLA), a fatty acid found in dairy and other bovine food products, improves clinical indicators of obesity related diseases, such as heart disease and diabetes, which makes this nutrient an ideal candidate for obesity prevention. Nutritional intervention studies have shown that consumption of 3.2g CLA per day is needed to achieve the health benefits stated above (Blankston et al., 2000; Shah et al 2013). However, bovine foods contain less than 1% CLA. Therefore, in order to obtain the 3.2g CLA needed to obtain a clinical response from these foods excessive amounts of saturated fat and cholesterol would also have to be ingested. Our laboratory has produced a 20% CLA-rich soy oil using patent pending processes (Proctor and Shah 2014; Chuan & Proctor, 2015).

Nutritional studies with genetically obese Zucker rats showed that supplementation with our CLA-rich oil for 3 months reduced total cholesterol by 41% and LDL cholesterol by 50% and reduced indicators of diabetes (Gilbert et al., 2011).

We are currently working with Riceland Foods to optimize CLA-rich oil synthesis for industrial production and are researching commercial uses of the oil. We have successfully produced CLA rich eggs, by adding CLA-rich soy oil to chicken feed. The CLA also increased egg quality in terms of yolk appearance, texture and shelf life (Shinn et al., 2015). Furthermore, we developed a CLA rich mayonnaise that delivers 1.8g CLA per serving using CLA-rich eggs and CLA rich soy oil (Shinn et al., in review); i.e. ~ 50% daily requirement.

The goal of this proposed research is to develop a commercially viable conjugated linoleic acid (CLA) rich scrambled egg-type product that would deliver the daily amount of CLA per serving needed to produce clinical effects.

This will be done using liquid and dehydrated CLA-rich eggs and CLA-rich oil. Drying CLA-rich eggs has the potential to increase the products shelf-life and transportability as well as improve ease of use and nutrition. Developing a production and processing protocol, determining shelf stability and

functional food properties are necessary for the commercialization of these egg products to be readily available to Arkansas.

Scrambled eggs and egg sandwiches are commonly consumed in Arkansas and USA (Tuder 2014). Using these foods as a vehicle to deliver CLA should be readily received by consumers. Daily consumption of these products has the potential to reduce serum cholesterol markers of diabetes indicators and potentially reduce obesity; e.g. reduce body fat (Shah et al 2013) and have a significant impact on the health of the state.

The objective of this project was to develop CLA-rich egg patties that are prepared either poached or fried in CLA-rich soy oil. These can be used for breakfast egg sandwiches and home use that will deliver the daily CLA requirement in one serving. The texture profile parameters and CLA content of these egg patties will be determined.

Materials and Methods

CLA-rich soy oil containing 20% CLA was prepared by the method of Proctor & Ruan (2015). Specifically, 0.5% Pricat Nickle (9925), 0.05% cysteine, 0.5% formic acid was combined with refined, bleached, degummed soy oil and processed for 3 h in a pilot scale hydrogenation unit at 200°C with 50 PSI pressure and 900 RPM mixing speed. In total 4 L of CLA-rich oil was prepared and homogenized. Table 1 includes the fatty acid and triacylglycerol profile of the CLA-rich and standard soy oil. Chicken feed was prepared with 10% CLA-rich oil, 10% conventional soy oil, and unmodified feed served as the standard control. The oil was added to standard commercial feed (Cobb-Vantress, Siloam Springs, AR) and mixed thoroughly using a Hobart stand mixer.

Two hundred white Leghorn chicks were reared under standard commercial conditions. At 24 weeks of age, 60 hens were randomly selected and assigned to single bird cages in blocks of 5 birds each, separated by 2 empty cages. All hens received the same standard commercial finisher diet ad libitum through 28 weeks of age. On the 29th week 3 blocks of 5 birds were assigned to either the standard control diet (STD), the control soy oil diet (SOY), or the CLA-rich diet (CLA) for a total of 45

birds. The remaining 15 birds were also split into the 3 treatment groups and served as spares. Birds were fed the diets at libitum for 48 days.

Egg preparation

Eggs were collected from day 12 until day 40, washed, labelled, and stored at 4°C until further processing. Eggs were broken open, homogenized using an immersion blender (Robot Coupe MP350 Turbo 14" Immersion Blender - 120V). The homogenate was filtered through three layers of cheese cloth to remove vitelline membrane remnants. Fresh egg homogenate was used for liquid egg patty preparation and analysis. Three liters of liquid eggs from each egg type were treated enzymatically by glucose oxidase and catalase to remove sugars that may cause browning during egg drying process. These eggs were used for egg drying and subsequent egg patty preparation and analysis.

Duplicate 1 g samples of each egg homogenate type were used for fatty acid analysis by GC-FID (Lall et al., 2008, Shinn & Proctor, 2013). Summaries of the egg fatty acid are summarized in Table 2. The enzymatically treated liquid eggs were dried in a bench top dehydrator (Excalibur Deluxe Black 5 Tray Food Dehydrator) at 41 °C for 6 hr in 200 mL aliquots on each tray. The dried egg cake was then ground using an electric coffee grinder. This preparation was done for each egg type in triplicate. Each type of egg powder sample were analyzed for peroxide value in duplicate.

To rehydrate the egg powder for subsequent cooking and analysis, approximately 120 g of egg powder was combined with distilled water in a small electric blender for 30 s intervals, and scrapping the sides of the cup with a spatula to insure complete mixture. Egg powders were rehydrated using the specific starting moisture content before dehydration (Table 1). The final mixture was ~160g. This was performed in duplicate for each egg type. Additionally, a commercial spray-dried egg product was rehydrated in duplicate and used for subsequent analysis as a commercial control (Ova Easy egg crystals).

Egg poaching

Eggs were poached in duplicate from each liquid and rehydrated egg sample (CLA, SOY, STD). An Excel steel 18/10 Stainless 6 Non Stick Egg Poacher was used to cook the eggs. One liter of

deionized water was added to the pan and set on a hot plate set to 200 °C. Approximately 35 g egg samples were weighed into the non-stick cooking cups and placed in the poacher. Eggs were covered and poached for 10 mins. The final egg patties were used for subsequent texture profile analysis. Egg patties were also used for duplicate lipid extraction to quantitate the final CLA content (Shinn & Proctor, 2013).

Egg frying in CLA-rich oil

Eggs were also fried in duplicate from each liquid and rehydrated egg sample (CLA, SOY, STD). The same pan and sample cups from the poached mentioned above was used to cook the eggs. The pan and cups were set on a hot plate set to 200 °C and the cups were heated to 350 °C, monitored by an IR temperature gun. Once the cups reached 350 °C, 4 g of CLA-rich soybean oil was added to each of the egg cups. The oil was heated to 300 °C and then the 30 mL of liquid egg was added. Eggs were fried for 5 minutes, where the egg was cooked through. Egg patties were also used for duplicate lipid extraction to quantitate the final CLA content.

Egg patty texture profile analysis

Texture profile analysis was performed on quadruplicate samples from each egg type and cooking type. This was performed using TA-XT2i Texture analyzer equipped with a 5 kg load cell and a 50 mm plate probe (Shinn et al., 2016). The probe was calibrated at 25 mm above the platform. The test speed was set at 3.2 mm/s and distance target mode was ½ the thickness of the egg patty.

Statistical analysis

All statistical analyses were performed using JMP 11 (SAS Institute, Inc., Cary, NC) statistical software. TPA parameters were analyzed by comparing the overall means by two-way ANOVA comparing effect of egg type and cooking method with an α -level of 0.05.

Results and Discussion

CLA content in poached and fried eggs

CLA-rich egg patties that were poached and prepared contained 140 mg of CLA per 100 g portion (approximately 2 eggs) on average. This was not different among powder and liquid samples. When the eggs were fried, there was 800 mg CLA in 4 g of CLA-oil that was initially added for frying. The eggs prepared from liquid homogenized eggs were better at absorbing the oil during cooking. 55% of oil was absorbed in the liquid eggs (460 mg of CLA) versus 17% in the powdered eggs (140 mg CLA). This means that the liquid CLA eggs fried in CLA oil would provide over 500 mg of CLA to a meal or breakfast sandwich. If this sandwich was prepared with a tablespoon of CLA-rich mayonnaise (Shinn et al., 2016) the meal would provide 2.8 g of CLA which is very close to the verified amount for health benefits ().

Texture profile analysis

Figures 1-3 show the significant findings from standard texture profile analysis on the prepared egg patties. Control liquid eggs fried in CLA oil were significantly harder and chewier than all other egg patty types. However, when control liquid eggs were poached, they were significantly softer and less chewy than all other egg types. In general, poached eggs were springier than fried eggs.

It is interesting to note that liquid eggs browned during frying while the powder did not. This is because before drying the eggs were enzymatically treated to remove sugars so no Maillard reaction occurred during frying. In addition, the liquid eggs would puff during frying and poaching, while eggs prepared from powder did not. Some protein must have been denatured during the drying process that aids in the puffing during cooking. Figure 4 shows the appearance of all prepared egg types. Figure 5 shows a breakfast sandwich prepared using a fried CLA egg patty and CLA-rich mayonnaise (Shinn et al., 2016), resulting in 2.8 g of CLA in the meal. These egg sandwich have the potential to be used in future human nutrition studies to determine the possible health benefits associated with eating CLA-rich food products.

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Tables and Figures

Table 1. Moisture content of homogenized eggs of three different types: CLA-enriched, soy-enriched control, and standard control eggs.

egg type	% moisture content	
	Mean	± Std Error
CLA	76.41	± 0.05
Control	74.62	± 0.06
Soy control	75.54	± 0.04

Table 2. Summaries of the fatty acid composition of each liquid egg homogenate.

Egg Type	Total CLA		Total SFA		Total MUFA		Total PUFA	
	Mean	± Std Error						
CLA	2.41	± 0.17a	57.18	± 1.77a	41.14	± 0.65c	27.37	± 1.97a
Soy control	0.00	± 0.00b	47.06	± 0.41b	51.99	± 0.75b	26.33	± 0.99a
Standard control	0.00	± 0.00b	47.36	± 0.38b	60.65	± 0.99a	17.82	± 0.88b

Figure 1. Hardness of egg patty samples define by standard texture profile analysis.

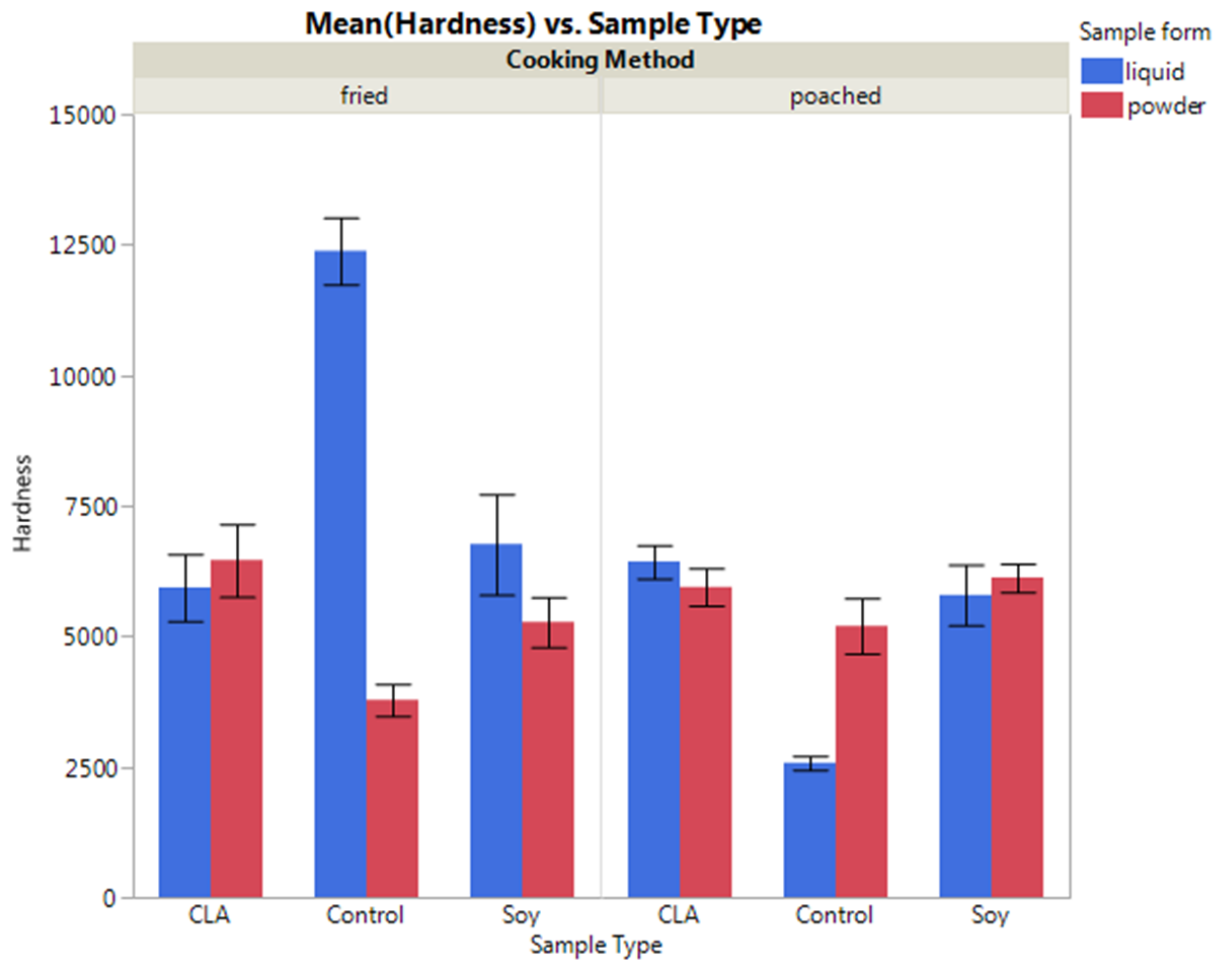


Figure 2. Chewiness of egg patty samples define by standard texture profile analysis.

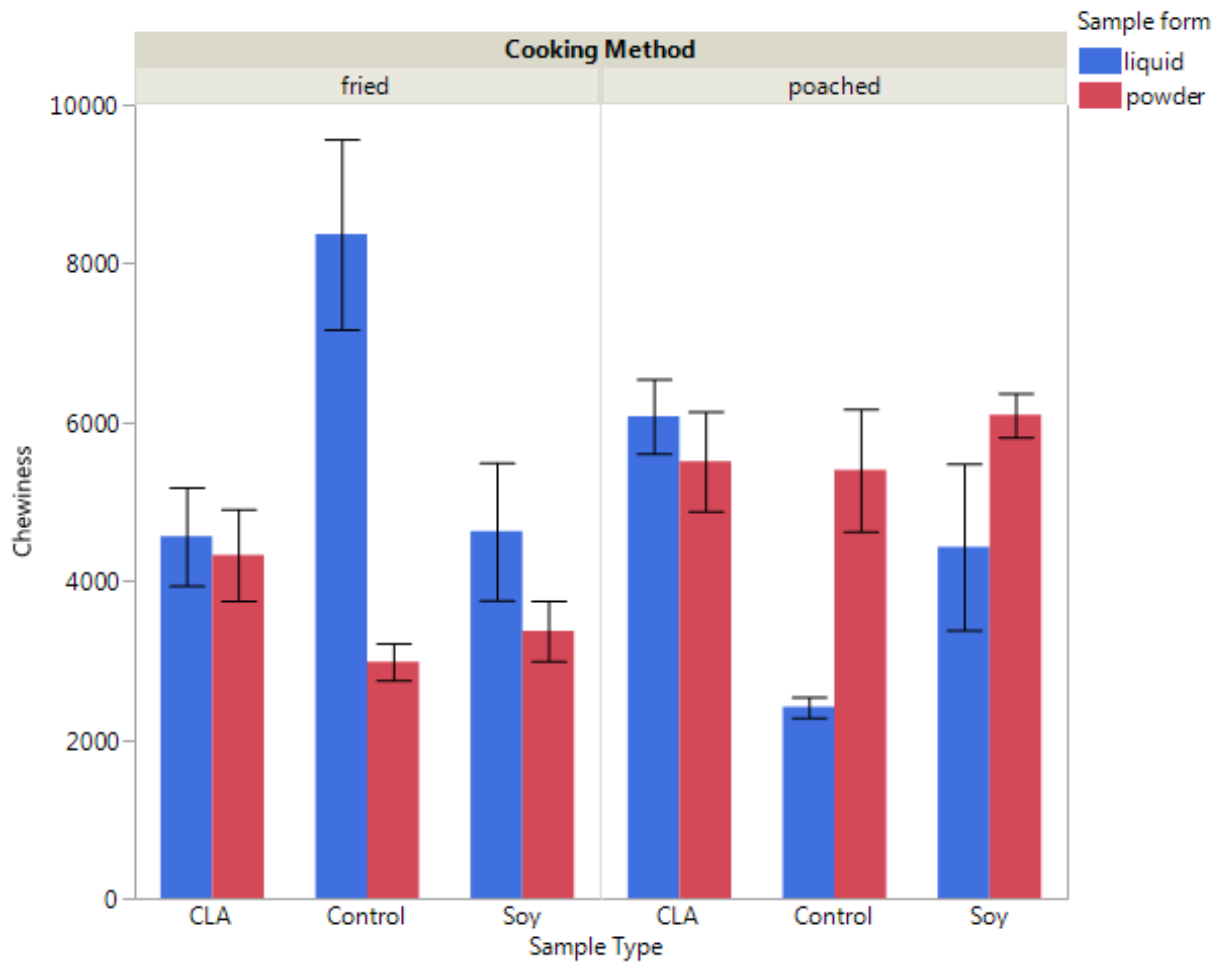


Figure 3. Springiness of egg patty samples define by standard texture profile analysis.

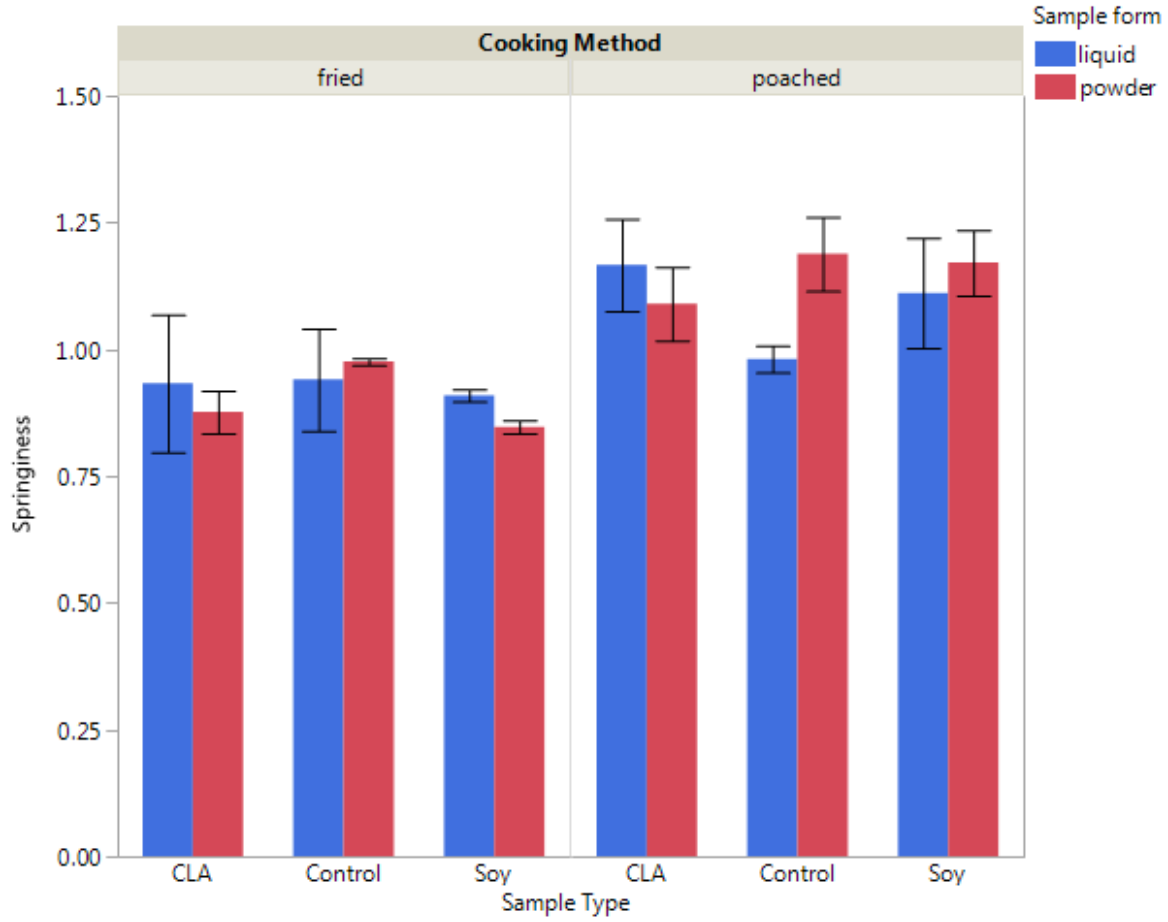


Figure 4. All prepared egg types.



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Figure 5. CLA-rich breakfast sandwich contain 2.8 g of CLA



CONCLUSIONS

Recent evidence has confirmed that diets containing greater amounts of healthy fats, like vegetable oils and fish, along with greater protein amounts, and reduced refined carbohydrates, reduce the risk of cardiovascular disease. CLA-rich eggs may be a diet-based approach to preventing or ameliorating the progression of CVD by combatting obesity. CLA and omega-3 consumption in Western diets may be increased by providing access to eggs enriched with one or a combination of beneficial fatty acids, or by utilizing these eggs as ingredients in other foods such as mayonnaise, pasta, salad dressings, or baked goods.

This collection of published manuscripts provides improved methods for egg lipid analysis (Chapters 2 & 3), data for the physiochemical properties of CLA-rich eggs (Chapters 4 – 7), and application of the CLA-rich eggs in food products (Chapters 8 & 9).

In summary, The CLA rich eggs with 120 mg of CLA had significantly more saturated fatty acids and less monounsaturated fatty acids than did the control eggs. Chapter 3 provided a comprehensive overview of intact lipid species and determined differences among CLA and commercially available eggs. Chapter 3 also illustrated that CLA enriched phospholipids and triacylglycerols are produced through the feed modification and may be isolated for further nutraceutical uses. This extensive yolk lipid profiling described how CLA incorporation affected other lipid species in the yolk. Providing the most comprehensive egg lipid profile to date may offer insight in further physiological and nutritional studies on CLA-rich and other designer eggs.

Chapter 4 describes the use of CLA-rich oil in the diets of three different hen strains. Overall, broiler hen CLA-rich egg yolks seemed to exhibit the most desirable fatty acid alterations with the lowest increase in saturated fat concentration, as well as significantly higher MUFA and PUFA levels, and the greatest CLA concentrations in yolks. This study suggests that CLA effects on other tissue FA composition may very well be strain and CLA-source dependent.

Chapter 5 assessed CLA-rich egg yolk quality relative to controls. Viscosity and vitelline membrane strength had not been previously reported in CLA eggs and filled a gap in knowledge for this field. Both determinations were greater than those of the control yolks and these qualities were preserved longer

throughout storage. These enhanced qualities may benefit egg shelf-life and provide advantages in prepared egg based dressings and sauces. The study did not find changes in CLA yolk weights or yolk indices, relative to controls. In contrast, other studies report CLA eggs had smaller yolk weights and larger yolk indices relative to conventional eggs, but storage conditions were either unspecified or different from those in this study. Literature on vitelline membranes is limited. However, Chapter 5 promoted the determination of vitelline membrane and proteins using sophisticated MS techniques (Chapter 6 & 7). Egg yolk viscosity in CLA eggs also seemed worthy of further study and was evaluated in mayonnaise applications (Chapter 8)

Chapter 6 was first report of vitelline membrane lipid modification in response to changes in the chicken's dietary lipids. The VM fatty acyl residue composition of CLA-rich yolks included CLA, greater SFA and significantly greater DHA as a result of CLA accumulation in the egg, relative to the standard control egg. Understanding how the VM ages, and how that can be modified by simple dietary adjustments may provide a means to develop more shelf-stable, longer lasting eggs to the marketplace. This report does support the idea that there are lipid composition modifications in the VM during storage, and this seems to have substantial effects on VM strength and integrity. In addition, changes in the VM lipid composition detected on the inner and outer membrane layers by direct MALDI-TOF-MS would not be apparent through traditional FAMES analysis by GC-FID. This research is a proof of concept that direct MALDI-TOF MS can be an invaluable tool for the identification of lipid species in biological membranes, without the need for lipid extraction and FAME derivation. This use of direct MALDI-TOF-MS to both inner and outer vitelline membranes show how applicable the technique is to accurate identification of intact lipid species, with less sample prep, and no need for prior lipid extraction.

Chapter 7 was a follow-up manuscript to validate how easily protein and lipid data can be collected using Direct MALDI-TOF-MS. The dominant peptides identified in the direct analysis of egg yolk vitelline membrane were 3 defensin-like peptides corresponding to m/z 4484, 4597, and 4778. The rapidity of MALDI to quickly determine distinct differences in vitelline membrane proteins as a result of CLA incorporation and storage is a valuable addition to egg literature

Chapter 8 evaluated how changing the fatty acid composition of egg yolk with CLA, and then using these enriched eggs in mayonnaise preparation: increased emulsion stability, reduced oil droplet size, and increased mayonnaise thickness and viscosity, relative to control yolk mayonnaise formulations. CLA-rich mayonnaise not only provides a value-added product, but it is also more viscous, retains its rigid structural appearance longer, and resists over-spreading and thinning, relative to mayonnaise prepared with control yolks.

Finally Chapter 9 developed a CLA-rich egg patty that can be used as a convenience food. A breakfast sandwich was prepared using a fried CLA egg patty (Chapter 9 Figure 4) and CLA-rich mayonnaise (Shinn et al., 2016, Chapter 8), resulting in 2.8 g of CLA in the meal. These egg sandwich have the potential to be used in future human nutrition studies to determine the possible health benefits associated with eating CLA-rich food products.

This dissertation provides a thorough, interdisciplinary evaluation of CLA-rich eggs. I hope it serves as a valuable resource for any future graduate student studying egg enrichment and egg analysis.

APPENDIX



MEMORANDUM

TO: Nicholas Anthony
FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee
DATE: February 25, 2013
SUBJECT: IACUC Protocol **APPROVAL**
Expiration date : **February 28, 2016**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol #13033 - "**Dietary effects of conjugated linoleic acid rich soy oil on egg yolk triacylglycerides, phospholipids and egg quality**". You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes to the protocol during the research, please notify the IACUC in writing [via the Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **02-28-2016**, you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian

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Publication: Journal of Agricultural and Food Chemistry

Publisher: American Chemical Society

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Author: Sara E. Shinn, Alex D. Gilley, Andrew Proctor, et al

Publication: Journal of Agricultural and Food Chemistry

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FUNDING AND GRANTS RECEIVED

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2013 Distinguished Doctoral Fellowship. University of Arkansas Graduate School. \$88,000.

TEACHING EXPERIENCE

FDSC 6133 Food Lipid Chemistry: Two invited lectures on 'Lipid analysis and characterization'. Department of Food Science, University of Arkansas. September 2014.

POSC 4314 Egg and Meat Technology: Invited lecturer for "Further Processed Egg Products: Gelation, Emulsion, and Foam Products." Department of Poultry Science, University of Arkansas, December 2014.

FDSC 4114 Food Analysis: Lecturer and Instructor in food total proximate analysis and food label preparation. Department of Food Science, University of Arkansas Spring 2015, Spring 2016

FDSC Honors undergraduate personal tutoring in research statistics August 2014 – May 2015.

PEER REVIEWED PUBLICATIONS

Doctoral publications:

Published:

Shinn, S. E., & Proctor, A. 2013. Rapid Lipid extraction from egg yolks. *Journal of the American Oil Chemists' Society*, 90 (2): 315-316.

Shinn, S.E., Liyanage, R., Lay, J., & Proctor, A. 2014. Improved fatty acid analysis of conjugated linoleic acid-rich egg yolk triacylglycerols and phospholipid species. *J. Ag. Food Chem.* 62 (28): 6608-6615.

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Shinn, S.E., Martin, E., Proctor, A. CLA-rich eggs in Mayonnaise: Emulsion stability and rheological properties. AOCS Annual Meeting, Orlando, Florida, May 2015.

Shinn, S.E., Liyanage, R., Lay, J., & Proctor, A. Isolation and Lipid Characterization of Chicken Yolk Vitelline Membranes. AOCS Annual Meeting, Orlando, Florida, May 2015.

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Shinn, S.E., Liyanage, R., Lay, J., & Proctor, A. A Method for Isolation and Lipid Characterization of Chicken Yolk Vitelline Membranes. AOCS Annual Meeting, Orlando, Florida, May 2015.

Shinn, S.E., Liyanage, R., Lay, J., & Proctor, A. Improved Fatty Acid Analysis of CLA-rich Egg Yolk TAG and PL Species determined by ESI-MS. AOCS Annual Meeting, San Antonio, Texas, May 2014.
Analytical Division Student Award.

Shinn, S.E., Gilley, A., Proctor, A., Anthony, N. Dietary effects of trans, trans rich conjugated linoleic acid soy oil on egg yolk lipids and egg quality: Preliminary trial with broiler-breeder hens. AOCS Annual Meeting, Montreal, Quebec, May 2013

Shinn, S.E., Proctor, A. Rapid Lipid Extraction from Egg Yolks. AOCS Annual Meeting, Montreal, Quebec, May 2013.

Shinn, S.E., Gilley, A., Proctor, A., Anthony, N. Dietary effects of trans, trans rich conjugated linoleic acid soy oil on egg yolk lipids and egg quality: Preliminary trial with broiler-breeder hens. **2nd Place in poster competition.** Ozark Food Processors Association, April 2013, Springdale, AR.

Measurement of Ions in Lake Brewer, Conway, AR. American Chemical Society Annual Meeting, February 2008, New Orleans.

INDUSTRIAL PRESENTATIONS

Shinn, S.E., Martin, E., Proctor, A. *CLA-rich eggs in Mayonnaise: Emulsion stability and rheological properties.*

Pepsi Co. Senior Principle Scientist visitation, Fayetteville, AR, August 4th, 2015

Shinn, S.E., Liyanage, R., Lay, J., & Proctor, A. *Isolation and Lipid Characterization of Chicken Yolk Vitelline Membranes*

Pepsi Co. Senior Principle Scientist visitation, Fayetteville, AR, August 4th, 2015

Shinn, S.E., Gilley, A., Proctor, A., Anthony, N. *Dietary effects of trans, trans rich conjugated linoleic acid soy oil on egg yolk lipids and egg quality: Preliminary trial with broiler-breeder hens.* Feed Energy Company, Pleasant Hill, Iowa, June 28, 2012.

PROFESSIONAL SOCIETY SERVICE

American Oil Chemist Society

Chair, Student Division 2013-present:

Symposium organized: *Progression of Your Career.* AOCS industry and academic professionals share insight on their Do's and Don'ts for Career paths. Annual Meeting, San Antonio, Texas, May 2014.

Symposium to be organized: *International Partnerships and Collaborations for AOCS students*. Resource meeting to facilitate international collaboration among AOCS students. Presentations by international faculty, students involved in international collaborations, and university administration. Salt Lake City, Utah, May 2016.

OUTREACH

Volunteer product developer and processor:

Food Innovation Center, Department of Food Science Department, University of Arkansas

Feed Fayetteville Gleaning Project. Summer 2013. Produce donated by the local farmer's market.

Fayetteville Public Schools Farm to Fork Tomato Sauce. Summer 2013.

Processed 3400 lbs tomatoes into 220 gallons of tomato sauce for use in public school cafeterias.

Green Fork Farms Fresh Salsa. Summer 2013.

660 16-oz jars sold in Fayetteville Farmer's Market and Ozark Natural Foods Grocer.

Sarah E. Shinn

Foundation Farms, Eureka Springs, AR, Summer 2013.

'Classic Basil Pesto' was produced and sold in local farmer's markets and groceries.

Arkansas Hunger Relief Alliance Grower's Gift Spaghetti Sauce. Summer 2013/2014.

Tomatoes from 'Arkansas Gleaning Project' processed to spaghetti sauce.

Proceeds from sales went to Hunger relief programs.