

Phospholipids oxidation and foaming enhancement of egg albumen

by

Guang Wang

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Program of Study Committee
Tong Wang, Major Professor
Donald C. Beitz
Lester A. Wilson
Stephanie Jung
Charles E. Glatz

Iowa State University

Ames, Iowa

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LIST OF ACRONYMS AND ABBREVIATIONS

³¹ P NMR	Phosphorus-31 nuclear magnetic resonance
AA	Arachidonic acid
ANOVA	Analysis of Variance
AOCS	American Oil Chemists' Society
BBEP	Bovine brain ethanolamine plasmalogens
BHT	Butylated hydroxytoluene
CSO	Commercial soybean oil
DHA	Docosahexaenoic acid
EDTA	Ethylenediaminetetraacetic acid
EL	Egg lecithin
ELSD	Evaporative light scattering detector
EPA	Eicosapentaenoic acid
EPL	Egg phospholipids
EthPm	Ethanolamine plasmalogen
FAME	Fatty acid methyl ester
F _c	Foaming expansion determined by the sparging method
FE	Foam expansion determined by the whipping method
FFA	Free fatty acid
FLS	Foam liquid stability determined by the whipping method
FS	Foam stability determined by the whipping method
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
HPLC	High pressure liquid chromatography
HTST	High temperature short time
IEC	Iowa egg council
K value	K value, foaming stability determined by the sparging method
LDL	Low density lipoprotein

LSD _{0.05}	Least significant difference at P< 0.05
MDA	Malondialdehyde
Meq	Milliequivalent
MSPI	Modified SPI
O/W	Oil-in-water emulsion
OSI	Oxidative stability index
PA	Phosphatidic acid
PC	Phosphatidylcholine
PDCAAS	Protein digestibility corrected amino acid score
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
pI	Isoelectric point
PLs	Phospholipids
PPM	Parts per million
PSO	Purified soybean oil
PUFA	Polyunsaturated fatty acids
PV	Peroxide value
ROS	Reactive oxygen species
SL	Soy lecithin
SMSPI	Sonicated-modified SPI
SPI	Soy protein isolate
TAG	Triacylglycerol
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid-reactive substances
TCA	Trichloroacetic acid
T _d	Denaturation temperature
TLC	Thin layer chromatography
TPP	Triphenyl phosphate

V_i	The speed of liquid being incorporated into foam determined by the sparging method
VLCFA	Very long chain fatty acid
VMD	Volume mean diameter
WPI	Whey protein isolate

CHAPTER 1. GENERAL INTRODUCTION

Rationale

Eggs are naturally nutrient-dense food that provides 13 essential nutrients in varying amounts and supports our daily activities. From 1997 to 2001, per capita egg consumption increased from 235 to 257, a level at which it has stabilized (Lawrence and others 2008). Broad application, high nutritional value, and efficient production of eggs drive egg industry to produce more raw eggs and value-added egg products. The egg industry is an important industry in the state of Iowa, because Iowa has been the largest egg producing state in the nation since 2001. While the distribution of shelled egg products is associated with high cost and potential quality deterioration over long distance transportation, increased needs have been expressed by the industry to conduct more research on developing value-added processing and egg products. Therefore, we received research fundings to address industry's need, as reported in this dissertation.

1. Egg lecithin utilization and oxidative stability in food emulsions

Commercial egg and soy phospholipid (PL) products are referred as lecithin. The name of lecithin originates from the Greek lekithos, which means egg yolk. Egg yolk contains 31.8-35.5% lipids on a dry weight base, and about 30% of the total lipids are PLs. PLs are an important component of biological membranes. They are present in all plant and animal products. Egg PLs or lecithin are mainly composed of phosphatidylcholine (PC, 66-76%) and phosphatidylethanolamine (PE, 15-24%) (Wang 2007). Egg lecithin has been extensively used in foods, pharmaceuticals, and cosmetics because it is a good emulsifier, and it is believed to be well-tolerated and non-toxic.

PLs have relatively more polyunsaturated fatty acids (PUFA) than do triacylglycerols (TAG) from the same source, especially arachidonic acid (AA, ω -6), eicosapentaenoic acid (EPA, ω -3), and docosahexaenoic acid (DHA, ω -3) (Pokorny 2003). Researchers have shown that PLs and PUFAs

provide multiple health benefits for infants, pregnant women, and the elderly (Canty and Zeisel 1994; Coouteaua and others 1997; Miller 2002; Uauy and Dangour 2006).

While PLs and PUFAs provide these nutritional and health benefits, the oxidative stability of PLs attracts increased interests because PLs can be susceptible to lipid oxidation. Lipid oxidation raises serious food quality and health concerns (Berliner and others 2001; Frankel 1998). The factors affecting PL oxidation include fatty acid composition, PL class composition, pH, temperature, oxygen exposure, transition metal ions, water activity, and the presence of antioxidants in foods, especially in oil-in-water emulsions (McClements and Decker 2000). An extensive study is presented in this dissertation to demonstrate the oxidative stability of egg lecithin and soy lecithin. They differ in fatty acid composition and PL class composition. Our hypothesis is that the difference in PL class makes each source of PL carry different charge under certain pH. The migration of transition metal ions to the lipid-containing droplets in emulsions will be affected by the pH change. Evaluation and comparison of the oxidative stability of PLs from different sources will provide information for specific applications.

2. Antioxidant effect of plasmalogens, a minor component found in egg lecithin

It was recently suggested that PL oxidation may be affected by another PL fraction in egg PL, e.g., plasmalogen. Palacios and Wang (2005) estimated the content of plasmalogens to be about 3.6% of total yolk PLs. Plasmalogens, a special class of PLs, are found in most PLs from animal sources. In the past few years, several comprehensive reviews have been published on the biological functions of plasmalogens, and special attention is given to its antioxidant effect *in vivo* (Nagan and Zoeller 2001; Brites and others 2004; McIntyre and others 2008). However, the debate on its pro- or anti- oxidant function is still not fully resolved. The reason for conducting our studies was to determine the role of plasmalogen in oxidative stability of neutral lipid and PLs. This study focuses on the antioxidant properties of plasmalogens from a classical perspective of lipid oxidation and analysis method

combined with other lipid oxidation design, including the liposome system. A relatively comprehensive conclusion is drawn based on our multiple oxidation design (bulk oil and liposome systems, with and without pro-oxidants) and with lipid analysis methods for both primary and secondary oxidation products.

3. Factors influencing egg white protein foaming and strategies for foaming enhancement

Egg albumen contains all essential amino acids and has the highest protein quality in terms of Protein Digestibility Corrected Amino Acid Score (PDCAAS). It also has excellent gelling, binding, and foaming functionalities and is widely used in various processed food. The application of liquid egg white in making meringues and angel food cakes is primarily attributed to its excellent foaming properties. However, industrially processed egg white inevitably has certain degrees of yolk contamination. Yolk contamination has detrimental effect on foaming performance. In addition to yolk contamination, thermal treatment and mechanical treatment encountered during industrial processing may also contribute to the damage in foaming. Therefore, the objectives of doing this study were: (1) to indentify the major lipid fraction in egg yolk leading to significantly impaired foaming of egg white. The result will help better understand the interaction of specific lipid fraction with proteins, therefore providing useful information for foaming enhancement; (2) to better understand the effect of industrial processing on foaming of egg white, especially yolk-contaminated egg white. This result will provide practical guide to egg industry in terms of optimal processing control; (3) to find suitable methods to improve the foaming of yolk-contaminated egg white. Various physical and chemical methods were used, and basic protein is proved to be an efficient and effective method. The hypothesis for the foaming enhancement of yolk-contaminated egg white is that basic protein partially neutralizes surface charge which the originally acidic proteins carry, therefore improving hydrophobic interactions among protein molecules for film formation.

Literature Review

Lipid oxidation overview

By a broad definition, lipids are a wide range of hydrophobic or amphiphilic molecules that can be extracted with certain organic solvents but not soluble in water; for examples, fatty acids or closely related derivatives or metabolites, fat-soluble vitamins (A, D, E, and K), waxes, sterols, and phospholipids (Frankel 1998). This dissertation mainly focuses on oxidation of phospholipids. Lipids contribute about 33% of the energy to a typical American diet. In comparison with proteins and carbohydrates (4 kcal per gram), lipids carry much denser energy (9 kcal per gram). Both nutritional and health benefit are provided by consuming lipid-containing food, especially those containing fat-soluble vitamins and ω -3 PUFA, a type of unsaturated fatty acids that have an end double bond in the third carbon position counting from the methyl end of the fatty acid.

Lipid oxidation has been recognized as a major cause for quality deterioration of fatty foods. Lipid oxidation in food normally happens either through autoxidation, photooxidation, or enzyme-catalyzed oxidation pathways. Lipid oxidation results in off-flavor (rancidity) and causes detrimental impact on other aspects of food qualities, such as vitamin retention, changes in smoke point and viscosity.

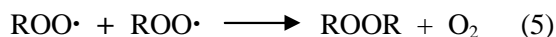
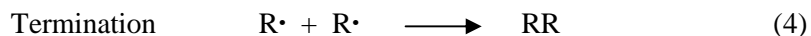
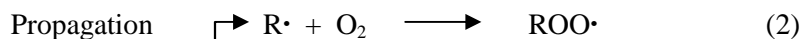
Phospholipids share the same glycerol backbone as triacylglycerol molecules except that the sn-3 hydroxyl group is esterified to a phosphoric acid, which has another group attached, such as choline, ethanolamine, inositol, or serine. PLs are minor components in plant lipids, but significant amounts are found in animal source. Currently, large quantities of PLs are derived as by-products of soybean oil refining, i.e., degumming (Jung and others 1989). Sunflower PLs are also seen great increase in supply. Egg PL is mainly used in cosmetic and pharmaceutical fields due to its high quality and high cost. PLs function as important emulsifiers in food matrices to facilitate emulsification and improve stability of heterogeneous mixture due to their amphiphilic nature. The

fatty acid composition varies with respect to PL source, but PUFAs tend to be richer in PLs than in TAGs for the same species (Pokorny 2003). The autoxidation of PLs is believed to bear a similar course to that of the simple fatty ester molecules. Recent studies have associated *in vivo* oxidation of PL with several diseases, such as atherosclerosis, inflammation, and heart disease (Berliner and Watson 2005). There has been an increased interest to maintain the benefits brought by consuming PLs, while minimize PLs oxidation when they are supplemented in diets.

PLs exist in food matrices, either in bulk oil or in dispersion with other food components. Because of the occurrence of other naturally existing antioxidants and pro-oxidants, the autoxidation course for PLs in specific system might be significantly different. It is valuable to review oxidation mechanism of lipid in various systems.

Mechanism of lipid autoxidation

The process of lipid autoxidation is classically described as three steps: initiation, propagation, and termination, as illustrated below (Kamal-Eldin and others 2003):



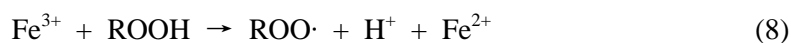
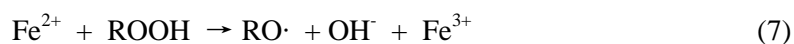
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Lipid oxidation from initiation step produces free alkyl radicals with the aid of an initiator. In food matrices, many factors could contribute to the occurrence of initiators, such as pre-existing

hydroperoxide and its dissociation products under thermal conditions; for instance, commercial soybean oil may contain detectable amount (2.4 meq/kg) of hydroperoxide (Tan and others 2002), and prolonged storage will cause even higher PV value. The hydroperoxides decompose in the presence of redox transition metal ions (iron, copper), and more alkyl radicals are produced for subsequent propagation (Chaiyasit and others 2007).

The abstraction of a hydrogen atom from the allylic or bis-allylic carbon is found to be the rate-determining step in the oxidation process (Kamal-Eldin and others 2003). Studies show that lipid oxidation rate of linoleic acid is about 20 to 40 times faster than oleic acid (Frankel 1998). With every bis-allylic methylene group increase, about a two-fold increase in oxidation is expected.

Hydroperoxide is relatively stable under mild temperatures (< 60 °C) in the absence of transition metal ions. Higher temperature leads to thermal decomposition of hydroperoxides, and in most situations transition metal ions greatly promote decomposition of hydroperoxide even under lower temperature, as illustrated below (Reische and others 2002; Berger and Hamilton 1995; Chaiyasit and others 2007):



Both copper and iron naturally exist in fats and PLs. Wang and Wang (2008) report that the content of copper in egg lecithin and soy lecithin is 2.6 and 1.2 ppm, respectively, and the content of iron is 3.4 and 11.4 ppm in egg and soy lecithin. The decomposition of hydroperoxide occurs through a homolytic or heterolytic cleavage mechanism (Frankel 1982). Homolytic cleavage involves an even splitting of paired electrons on hydroperoxide group, whereas heterolytic involves hydrogen and water molecules' participation under an acidic condition. Further splitting of hydroperoxide produces a series of secondary oxidation products, including alkenes, ketones, aldehydes, short chain fatty acids, and even polymers under severe conditions or prolonged storage (Chaiyasit and others 2007).

PLs oxidation in oil-in-water emulsion

Fats and oils can exist as either minor or major components in heterogeneous food matrices, but, in most cases, PLs mainly function as emulsifiers. PL oxidation in emulsion systems involves interfacial phenomena that are affected by the pro-oxidant and antioxidant activities (McClements and Decker 2000). Oxidative stability of PLs in emulsions becomes more difficult to control than in bulk oils, not only because of fatty acid composition, but also because of the amphiphilic nature of PLs. PLs tend to stay at the interface of the oil-in-water emulsions and expose large surface area to an aqueous phase, therefore are in contact with the solubilized oxygen in water, reactive oxygen species (ROS), and other pro-oxidants, such as pigments, free radicals, and transition metal ions.

Transition metal ions are naturally occurring in food emulsions and are the major pro-oxidants catalyzing hydroperoxide decomposition. Copper attracts less interest than iron, but it is reported to be as effective as iron in promoting lipid oxidation, though its content is usually lower than iron (Yoshida and Niki 1992). Copper-induced lipid oxidation also follows the Fenton reactions as illustrated in equation (7) and (8) as for the iron. Considering the fact that PLs are either anionic or zwitterionic, whereas transition metal ions are positively charged, it is reasonable to predict that pH environment in the continuous phase would significantly contribute to the PL oxidative stability due to opposite charge interactions. Because PLs from different sources have different PL classes, the oxidative stability is expected to be different as well (Wang and Wang 2008). For example, egg lecithin contains mainly zwitterionic PE and PC, whereas significant amounts of anionic phosphatidylinositol (PI) and phosphatidic acid (PA) are present in soy lecithin.

Besides fatty acid composition, PL class, and transition metal ions, there are many other factors contributing to the PLs oxidation in emulsions, such as oxygen concentration, availability of antioxidants, and physical characteristic of the emulsion (Wang and Wang 2008). Comprehensive

reviews are given by McClements and Decker (2000) and Chaiyasit and others (2007) in terms of common fats and oils, and these in general will apply to PLs oxidation.

Recent progress on the study of plasmalogen, a controversy over anti-oxidation or pro-oxidation

Plasmalogens are a special class of choline- and ethanolamine-containing PLs that share the common structure of normal PE and PC except that they contain a vinyl ether moiety at the sn-1 position of glycerol backbone (Glonek and Merchant 1996; Wang and Wang 2008). Significant amounts of plasmalogens are found in brain, testes, kidney, heart, and skeletal muscles (Brites and others 2004; McIntyre and others 2008). It is common that the sn-2 position of plasmalogens is preferentially esterified with DHA and AA, and saturated or moderately unsaturated fatty acids tend to attach on the sn-1 position (McIntyre and others 2008). The vinyl ether bond is alkaline stable but extremely susceptible to acid hydrolysis (Leßig and Fuchs 2009).

Plasmalogens, especially ethanolamine plasmalogen (EthPm), are reported to carry important biological functions *in vivo*, including membrane fluidity and signal transduction. Zoeller and others (1988) suggested that plasmalogens might function as antioxidants in photosensitized oxidation in animal cells. The acid-labile vinyl ether bond allowed the plasmalogen to scavenge ROS (Hahnel and others 1999) based on the fact that the hydrogen atoms at α -carbon next to the vinyl ether bond have relatively low bond dissociation energy (Leßig and Fuchs 2009). Engelmann and others (1994, 2004) claimed that EthPm showed certain antioxidant effects, and one vinyl ether double bond prevented four double bonds in an arachidonate from its oxidation.

Researches also have been conducted towards understanding the interaction between plasmalogen and transition metal ions. Zommara and others (1995) demonstrated that in liposome systems that contained egg choline PLs, the EthPm inhibited iron- and copper-promoted peroxidation in the presence of hydroperoxide initiator. Similar results were reported by Sindelar and others (1999)

who found that brain plasmalogens effectively protected iron-induced lipid peroxidation. The mechanism of action at the molecular level supporting the plasmalogen-mediated protection to transition metal-induced lipid peroxidation is still not clearly defined, though a binding stoichiometry of 1:1 for copper ion: vinyl ether was claimed (Hahnel and others 1999).

On the other hand, there are different observations indicating that no antioxidant effects were demonstrated by plasmalogens (Yavin and Gatt 1972; Fauconneau and others 2001; Farooqui and others 2008). The breakdown of the vinyl ether bond was observed following oxygen consumption, and the degradation of plasmalogens became faster when highly unsaturated fatty acids were at the sn-2 position. Rancidity development in meat products also might be attributed to the instability of plasmalogen (Marmer and others 1986). Further evidence showed that α -hydroxyaldehydes, a class of decomposition products of plasmalogen epoxidation, were detected, and their high reactivity also raised doubts on the protective effect of EthPm in biological systems (Loidl-Stahlhofen and others 1995; Felde and Spiteller 1995). It is interesting and needed to test this class of polar lipids in lipid oxidation and liposome systems.

Egg albumen foaming and its interaction with yolk lipid

Protein foam is a colloidal dispersion system. During foaming soluble proteins first rapidly migrate to the air-water interface with the aid of energy input, such as whipping and air sparging, followed by protein denaturation (unfolding) and rearrangement of protein molecules. Polypeptide interactions mainly contribute to the formation of cohesive and visco-elastic film (Zayas 1997). Another function of proteins in foam formation is to decrease the interfacial tension due to their amphiphilic nature. However, surface tension does not change proportionally to foaming power (Kitabatake and Doi 1982). A good protein foam is a combined result of multiple factors, including protein type (reflecting protein net charge, hydrophobicity, and flexibility), concentration, solubility,

pH, ionic strength, temperature, beating method, and other additives, especially the presence of foam inhibitors that prevent the formation and stabilization of foams.

Egg albumen is extensively used in processed foods because of its excellent foaming properties, especially in ice cream, meringue, and angel food cake (Macherey 2007). Egg albumen contains more than 40 types of proteins, among which seven types represent the majority as listed in Table 1 (Alleoni 2006). As observed from the isoelectric point (pI) of individual protein fractions, most fractions are acidic, and lysozyme is the only basic protein with a significant amount in egg white. Egg white foaming has been studied extensively in numerous model systems by using single albumen fractions, and useful information was obtained regarding the specific functions of each protein fraction in foaming performance. However, Lechevalier and others (2004) confirmed that the information obtained from such single fractions cannot easily extrapolate the native complex of egg white.

Several types of interactions involved in protein foaming are proposed, including electrostatic, hydrophobic, steric interaction of the protein molecules, and disulfide bonding. The electrostatic repulsion can reduce foam stability and delay the film formation, whereas disulfide bonding reduces protein flexibility and, therefore, it was suggested to minimize the repulsion to achieve stable foam (Nakai 1983; Zayas 1997).

Yolk lipid is a major foam inhibitor for egg white. The extent of yolk contamination may vary from 0.01% to 0.2%, depending on operational conditions and quality of raw egg white (Cotterill and Funk 1963; Stadelman and Cotterill 1995; Wang and Wang 2009). Even with 0.01% contamination, egg white showed significant reduction in the foam expansion. Yolk contamination, especially with neutral lipids, showed significantly more reduction in foam stability than the phospholipid fractions (Wang and Wang 2009). Yolk contamination might lead to local structural rupture in the film and cause bubble collapse. It is believed that foam damage caused by yolk

contamination arises from the weakened protein-protein interactions by lipid interruptions at the hydrophobic interfaces.

However, there has been a controversy over which lipid fraction causes the foam damage of contaminated egg white. Smith (1959) and others (Cotterill and Funk 1963; Lomakina and Mikova 2006) reported the detrimental effect caused by the addition of neutral lipids, whereas Damodaran (1996) and others (Zayas 1997; Alzagat and Alli 2002) claimed that the damaged effects were caused by the addition of the lecithin to egg white and whey protein systems. There is a need to clarify the role of neutral and polar lipid in foaming reduction.

Foaming enhancement of yolk-contaminated egg white protein

The application of basic proteins to improve protein foaming was first studied by Poole and others (1984) who discovered that clupeine (pI of 12) and lysozyme (pI of 10.7) did not form stable foams when used alone. However, adding each of these two proteins to several acidic proteins greatly improved the protein foaming, with clupeine being more effective in promoting foaming than lysozyme at the same concentration. And clupeine significantly improved foaming properties of egg yolk-contaminated egg white (Poole and others 1986). It is obvious that the charge of basic proteins is an important factor for foaming enhancement. It was hypothesized that addition of basic protein neutralizes the charge of proteins, thereby increasing hydrophobic interactions of protein molecules (Wang and Wang 2009).

Table 1. Physical-chemical characteristics of albumen proteins (source: *Li-Chan and others 1995; Johnson 1994*)

Protein	Relative weight %	pI	MW, KDa	Td	SH	-S-S-	Characteristics
Ovalbumin	54.0	4.5	44.5	84	4	1	phosphoglycoprotein
Ovotransferrin	13.0	6.1	77.7	61	0	15	Glycoprotein, binding metal ions
Ovomucoid	11.0	4.1	28.0	77	0	8	Glycoprotein, trypsin inhibitor
Ovomucin	3.5	4.5-5.0	5.5-8.3x10 ³	-	-	9?	glycosulphoprotein, sialoprotein
Lysozyme	3.4	10.7	14.3	75	0	4	Lyses bacteria cell wall
Globulin G2	4.0	5.5	49.0	92	-	-	
Globulin G3	4.0	5.8	49.0	-	-	-	
Avidin	0.05	10.0	68.3	-	-	-	binding biotin

T_d: denaturation temperature (°C)

In reality, clupeine and lysozyme are rarely found in abundance in nature (Poole 1989). Clupeine is currently obtained from herring roe, and lysozyme is from egg white as a minor component (3.4%). As a matter of fact, it was shown that chemical modification to make basic protein is attractive because the reaction is relatively easy and well defined (Means and Feeney 1971). To show better functionality in foaming, such modified protein usually requires a pI value higher than 9 (Poole 1989). Wang and Wang (2009) tested yolk-contaminated egg white by adding methylated soy protein isolate (pI of 9.5), and they found that both foaming expansion and foaming stability were enhanced, and they were even restored to the level of yolk-free egg white. This method is expected to be extended to other protein sources and with alternative alcohol derivatives for wider application in food.

Dissertation Organization

This dissertation contains a general introduction, including a research rationale and a literature review, followed by four research papers and a general conclusion. The papers are in the required corresponding journal formats.

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CHAPTER 2. OXIDATIVE STABILITY OF EGG AND SOY LECITHIN AS AFFECTED BY TRANSITION METAL IONS AND PH IN EMULSION

A paper adapted from the publication in the *Journal of Agricultural and Food Chemistry*¹

Guang Wang² and Tong Wang^{2, 3}

Abstract

The oxidative stability of egg and soy lecithin in emulsions was evaluated with two transition metal ions, cupric and ferric ions, at two concentrations (50 and 500 μM). The effect of pH on lipid oxidation was also examined under these two concentrations for each ion. Egg lecithin (EL) had a similar peroxide value (PV) development pattern to soy lecithin (SL) when treated with cupric ion under both acidic and neutral pH. Acidic pH of 3 accelerated the oxidation of both EL and SL, especially with high concentration of copper. When treated with ferric ion, EL oxidized much faster than SL did. EL had higher values of thiobarbituric acid-reactive substances (TBARS) than SL, possibly because of its higher content of long-chain polyunsaturated fatty acids (PUFA). Acidic pH accelerated TBARS development for both EL and SL, but EL had more significantly increased values. Cupric ion was more powerful than ferric ion in catalyzing oxidation of both EL and SL under both acidic and neutral pH conditions as measured by TBARS. Linoleic acid may contribute to higher PV production. However, arachidonic acid and docosahexaenoic acid may have contributed more to TBARS production. Overall, SL showed better oxidative stability than EL under the experimental conditions. This study also suggests that using multiple methods is necessary in properly evaluating lipid oxidative stability.

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² Department of Food Science and Human Nutrition.

³ Author for correspondence.

KEYWORDS: Egg lecithin; emulsion; fatty acid composition; lipid oxidation; oxidative stability; pH effect; phospholipids; soy lecithin; transition metal ions

Introduction

Lipid oxidation and the generation of secondary oxidation products have always been serious concerns of food quality and consumer health (1). Studies have shown that fatty acid composition is the dominant factor affecting lipid oxidative stability in bulk oil and emulsions (2-4). Several researchers reported that the lipid oxidation rate of linoleic acid was 20 to 40 times faster than that of oleic acid (5-7). In many food emulsions, n-3 and n-6 polyunsaturated fatty acids (PUFAs) represent significant amounts of the fatty acids in total lipid because of their natural presence and in fortified formulations. The rancidity development of such foods is much faster compared with that of formulas containing more saturated lipids.

Transition metal ions commonly found as copper and iron in food emulsions, are major pro-oxidants that catalyze lipid oxidation. Copper has received less interest than iron because of its lower content in food, but it was reported to be as effective as or even more active in accelerating the decomposition of primary oxidation products (8). For each ion, the cuprous and ferrous ions are much more effective than the cupric and ferric ion in catalyzing oxidation, but the ions with higher oxidation state are more stable.

There are other important factors that contribute to lipid oxidative stability in emulsions, such as oxygen availability, storage temperature, and pH. These topics have been reviewed thoroughly (2).

Phospholipids (PLs) or lecithin are typically used as food emulsifiers because of their amphiphilic characteristic. Different PLs may have different oxidative stability not only because of fatty acid composition but also because of their PL class composition. Regarding fatty acid

composition, egg PLs or egg lecithin (EL) contains less linoleic and nearly no linolenic acid but more long-chain polyunsaturated fatty acids (PUFA) than soy lecithin (SL), whereas SL has mainly linoleic acid and a low amount of linolenic acid. In terms of PL class, EL contains mainly zwitterionic phosphatidylethanolamine (PE, 18.1%) and phosphatidylcholine (PC, 78.7%), whereas a relatively high levels of anionic PLs, such as phosphatidylinositol (PI, 17-24%) and phosphatidic acid (PA, 6%), are also present in SL, along with 25.7% PE and 35.3% PC (9). The difference in the electric charge of PLs was hypothesized to contribute to their oxidative stability, especially in emulsions and under different pH conditions. It is believed that negatively charged emulsifiers will attract positively charged transition metal ions, and thus such action stimulates the formation and decomposition of lipid hydroperoxides. It is unknown how the oxidation of EL and SL as PL mixtures is affected by the two transition metal ions under different pH environments. A preliminary study conducted in our research showed that cupric ion did not accelerate oxidation of EL in emulsion, but they did accelerate oxidation of SL in emulsions when the concentration of cupric ion was only 1 ppm.

The objectives of this research were to determine how oxidative stability of egg and soy lecithin is affected by type and amount of transition metal ions, i.e. cupric and ferric ions, in emulsions, as measured by the formation of primary and secondary oxidation products; and to evaluate the effect of pH of emulsions on the oxidative stability of lecithins in the presence of transition metal ions.

Materials and Methods

Materials. EL was purchased from Q.P. Corporation (Tokyo, Japan) with claimed purity of near 100%. SL was obtained from Fisher Scientific (Fair Lawn, NJ) with purity of 99%, and it was further washed using acetone (AOCS Official Method JA 4-46) to remove neutral lipids and residual tocopherols. Mineral oil was obtained from Fisher Scientific (Fair Lawn, NJ). Cupric sulfate, ferric chloride, isooctane, 2-propanol, methanol, chloroform, 2-thiobarbituric acid, hexanes, and other

reagents were all purchased from Sigma-Aldrich (St. Louis, MO).

Lecithin Characterization. Initial peroxide values (PV) of both EL and SL were determined by using a modified ferrous ions method as described by Wang et al (10). Briefly, slightly oxidized soybean oil, with PV 7.45 meq/kg as measured by the AOCS Official Method Cd 8-53 (11), was used to establish a standard curve for the quantification of the lipid oxidation. Lecithin, 10 mg, was dissolved in 5 mL of chloroform: methanol (7:3, v/v). Ammonium thiocyanate solution (15 μ L, 3.75 M) and freshly prepared ferrous chloride solution (15 μ L, approximately 0.014 M) were added to the test tube, and the mixture was stirred well. After 10 min, the absorbance at 500 nm (for the purple-red color) was measured by a Genesys 20 spectrophotometer (Cambridge, U.K.), and PV was calculated using the standard equation.

Determination of natural cupric and ferric ion content in two lecithins was conducted by following the atomic absorption spectrometric method of AOAC 990.05 (12). To prepare samples for analysis, five grams of lecithin samples were ashed in a Thermolyne 1400 laboratory furnace until white ash formed. The ash was then dissolved in 10 mL of nitric acid (10%). The samples were then analyzed at the Soil & Plant Analysis Laboratory at Iowa State University.

To measure tocopherol content in both lecithin samples, a HPLC method modified from AOCS official method Ce 8-89 (11) was used. Lecithin, 5 g, was first subjected to saponification following AOCS official method Ca 6a-40 (12), and the unsaponifiable matter was extracted. The extract was used for HPLC quantification for tocopherols. Alpha-, gamma-, and delta-tocopherols purchased from Sigma-Aldrich were used as external standards for the quantification.

The lecithin fatty acid profile was determined by using a Hewlett-Packard model 5890 series II gas chromatograph (GC) with a flame ionization detector. Ten mg of lecithin was first dissolved in 4 mL of sodium methoxide (1 M in methanol). The reaction was conducted for 30 min under ambient temperature (25 °C) and was stopped by adding a few drops of water. Fatty acid methyl esters

(FAME) were extracted with hexanes and applied to GC for analysis. The conditions used for GC were as follows: injection temperature, 230 °C; detector temperature, 230 °C; oven temperature was programmed from 110 to 220 °C with heating rate of 10 °C/min. The column used was a Supelco SP-2330 (Bellefonte, PA) capillary column, 15 m (length) \times 0.25 mm (i.d.) \times 0.2 μ m (film thickness). In addition, fatty acid profile of each PL class was measured by first using thin layer chromatography (TLC) to separate PL classes, and then using the GC method to determine fatty acid composition after the PL bands were transesterified as described above. The conditions used for TLC were as follows: TLC plate, Alltech Adsorbosil Plus 1 with dimensions 20 \times 20 cm, 500 μ m (Deerfield, IL); the mobile phase was chloroform: methanol: water (25:10:1, v/v/v).

The PL class composition was determined by an HPLC method, as described by Wang et al (13). Briefly, a Beckman-Coulter Gold HPLC system equipped with a model 508 autosampler and model 126 delivery pumps was connected to an Alltech 2000 evaporative light scattering detector (ELSD). A Pholipidec normal phase silica column (250 mm \times 4.6 mm i.d., 5 μ m particle size) with an integrated guard column was used for lipid separation (Advanced Separations Technologies, Whippany, NJ). A gradient program with two mobile phases at a flow rate of 1 mL/min was used. Phase A was chloroform/methanol/ammonia (80:19:1, by volume) and phase B was chloroform/methanol/ ammonia/water (50:48:1:1, by volume). Phase B increased to 100% during the first 25 min and was maintained there for 15 min, then returned to 0% in 2 min. Phase A stayed for another 6 min at 100% before the next analysis was run.

Emulsion Preparation and Sampling. All glassware was immersed in a nitric acid solution (10%, v/v) for 4 h and then rinsed with deionized water before use to ensure all glassware free from metal ions contamination. Oil-in-water (30%, o/w) emulsions were prepared by the following procedure: lecithin, 4.5 g, was dispersed in 45 mL of mineral oil under 45-50 °C heating. Cupric sulfate and ferric chloride were pre-dissolved in deionized water with concentration of 500 μ M.

Amounts of each solution were added to the water phase to obtain the desired concentrations of 0, 50, and 500 μM . Emulsions were prepared by using a Polytron homogenizer equipped with a 3012/2T generator at a speed of 20,000 rpm for 60 s in an ice-water bath. Samples from all treatments were stored in a dark oven at 60 °C for 25 days. Ten mL of samples were taken at day 0, 2, 6, 10, 15, 20, and 25. All treatments were re-homogenized every 24 h and before each sampling. All samples were stored in a -20 °C freezer for further lipid extraction and analysis.

Emulsion Droplet Size Analysis. Particle size distribution of the oil droplets was measured immediately after emulsion preparation by using a Hydro 2000 MU Laser Scattering Particle Size Analyzer (Malvern, U.K.). The result was expressed as the volume mean diameter (VMD) $D[4,3]$, which measures the average diameter based on the particle volume and is calculated as $D[4,3] = \frac{\sum n_i d_i^4}{\sum n_i d_i^3}$. To measure $D[4,3]$, emulsions (about 0.2-1 mL) were placed into 1 L of water with stirring until the obscuration (%) reached a level between 10 and 15, which indicates an ideal count of particles for an accurate measurement. The final concentration of the emulsion was estimated as between 0.002% and 0.03%. Each sample was measured three times, and results were presented as mean values.

Zeta Potential. The surface charge of the oil droplets, as expressed by zeta potential, was measured for each sample right after the emulsion was made. Zeta potential characterizes electric potential difference between the charged emulsion droplet and the surrounding continuous liquid phase. A Malvern Zetasizer Nano Z potential (Malvern, U.K.) was used. Each sample was diluted 1000 times, well mixed, and placed into a conductive cell. Each sample was prepared twice for this measurement, and each preparation was measured three times to obtain the mean values.

Lecithin Oxidation Quantification. Ten mL of emulsion containing 300 mg of lecithin and 3 mL of mineral oil were added to 40 mL of a mixture of isooctane/2-propanol (3:1). Each sample was vortexed and mixed thoroughly for lipid extraction, followed by centrifugation for 5 min at

1000g (14). The organic phase was rotary evaporated at a temperature below 45 °C, and the residual solvent was removed by using a vacuum oven overnight. All samples were filled with nitrogen and stored in a -20 °C freezer until further analysis.

To measure PV change with time, the colorimetric method mentioned in lecithin characterization was used. An oil sample of 100 μ L containing 10 mg of lecithin was used. To measure secondary oxidation products, a method determining the amount of 2-thiobarbituric acid-reactive substances (TBARS) modified from references (15, 16) was used. Briefly, 15% (w/v) trichloroacetic acid (TCA) and 0.375% (w/v) thiobarbituric acid (TBA) were dissolved in 0.25 M hydrochloric acid aqueous solution by mild heating and agitation. Butylated hydroxytoluene (BHT), 3 mL of 2% in absolute ethanol, was added to 100 mL of the TCA/TBA stock solution. An oil sample of 400 μ L containing 40 mg of lecithin was added to 4 mL of TCA/TBA stock solution in a test tube. The mixture was mixed thoroughly with a Vortex mixer, and it was put in a boiling water bath for 15 min, and then cooled to room temperature. All samples were then centrifuged at 1000g for 5 min. TBARS were measured at 535 nm with a blank containing only TCA/TBA reagent and mineral oil of 0.4 mL. Concentrations of TBARS were determined using a standard curve prepared using 1, 1, 3, 3-tetraethoxypropane.

Duplicate assays were used for each treatment for both PV and TBARS determinations.

Statistical Analysis. Data analyses were done by using SAS program (version 9.1, SAS Institute Inc., Cary, NC). One-way analysis of variance (ANOVA) was used, and Fisher's least significant differences were calculated at $P < 0.05$.

Results and Discussion

Lecithin Characterization. The initial oxidation state of lecithin is important because it can affect the rate of further oxidation of lipids. The PV of EL was 0 measured by the AOCS official method, and it was 0.054 meq/kg measured by the modified iron colorimetric method. The PV of SL

was 2.29 and 4.91 meq/ kg by these two methods. The results from these two quantification methods showed that the colorimetric method gave slightly higher values than the AOCS method. Because we had a very limited amount of PLs for oxidation evaluation in emulsions, and the colorimetric method is a highly sensitive and convenient method to use, we chose this method for PV quantification. It was determined that evaluating oxidation trend was more important in this study than determining the absolute value of lipid oxidation; therefore, the slight difference between the two methods was not a serious concern for this study. Nonetheless, the difference in PV was suspected to be caused by the color difference when the oil and PL hydroperoxides reacted with the reagents.

Elemental iron and copper in EL were 3.4 and 2.6 ppm, and they were measured as 11.4 and 1.2 ppm in SL.

Tocopherol quantification by HPLC analysis detected no tocopherols in EL, whereas three tocopherols, alpha, gamma, and delta, existed in the commercial SL, and their contents are presented in **Table 1**. It is shown that considerable amounts of gamma (about 11 mg/100 g) and delta (about 13 mg/100 g) tocopherols were contained in the commercial SL. Acetone washing removed 96% of these tocopherols.

Fatty acid profiles of both EL and SL are shown in **Table 2**. EL contained much less linoleate, 16.2%, and linolenate (not detectable) compared with SL which had 56.6% and 7.1% of these fatty acids. On the other hand, EL had significant amount of other PUFA, such as arachidonic acid (AA, C20:4 ω 6, 6.1%), and docosahexaenoic acid (DHA, C22:6 ω 3, 1.7%). Fatty acid identification from C14 to C18 was verified by standard samples from Nu-CHEK-PREP. Inc. (Elysian, MN). C20:4 and C22:6 were confirmed by comparing to EL specification and analytical report provided by our supplier. The long chain PUFAs totaled to 9.4% in EL. All fatty acids were relatively uniformly distributed in PC and PE fraction of SL. For EL, higher amount of AA was found in PE than in PC, and its PE fraction had less oleic, linoleic, and palmitic but more stearic acid

than PC. HPLC analysis showed that PC/PE ratio in EL was 4.3, whereas it was 1.4 for SL. The total quantifiable PLs in SL were 61%. SL contained a significant amount of PI too, but it was not quantified because of some unexpected technical difficulties.

Effect of pH and Cupric Concentration on PV Development of EL and SL. For EL and SL oxidation in emulsion without any metal ion addition, no differences were observed for types of lecithin and under the two pH conditions, as shown in **Figure 1**. All four treatments were slowly oxidized, and PV reached about 40 meq/kg at the end of 25 days.

To examine if pH affects emulsion stability, particle size of the oil droplets was measured. It was found that, under acidic pH, neither EL nor SL emulsion was as stable as they were under neutral pH as shown in **Table 3**. The particle sizes of EL control under neutral and acidic conditions were 6.2 and 53.2 μm , respectively, and they were 8.9 and 253.4 μm for SL control. And for all the treatments with metal ions added, the particle diameter values under acidic conditions were all much higher than those under neutral conditions. The reason for this observation is unknown.

Addition of 50 μM (about 3.2 ppm) cupric sulfate did not change overall trend of PV for all treatments, though SL showed slightly higher PV than EL under pH 7 and 3 (**Figure 2A**). pH did not show a significant effect for both EL and SL at this ion concentration. This slight more oxidation of SL than EL at low cupric concentration agrees with our previous research results where 1 ppm cupric under neutral pH was used to compare oxidative stability of the two lecithins. As the concentration of cupric sulfate was increased to 500 μM (32 ppm), pH effect became significant. For EL and SL emulsions, low pH significantly increased degree of oxidation of PLs as shown in **Figure 2B**. PVs of low pH EL and SL quickly increased to above 40 meq/kg after 10 to 15 days, and PVs of neutral pH EL and SL were less than 20 meq/kg at the same time. Under both pH conditions, EL had the similar oxidative stability to SL. Therefore, at high cupric concentration, EL and SL were oxidized faster at acidic pH than at neutral pH, and the two lecithins behaved similarly for their oxidative stability

under the two cupric and two pH conditions, as measured by lipid hydroperoxide formation.

To illustrate PV's response to cupric concentration under acidic pH, we plotted **Figure 3**, which shows the EL oxidation. It seems that as cupric concentration increased to 500 μM , the induction period for EL oxidation was shortened.

Effect of pH and Ferric Concentration on PV Development of EL and SL. Different from cupric treatments, for treatments with 50 μM (2.8 ppm) ferric chloride, both EL and SL at pH 3 oxidized slightly faster than that at pH 7 at early stage of the oxidation, as shown in **Figure 2C**. In addition, under both pH conditions, EL started to show higher PV than SL after day 10. For treatments containing 500 μM (28 ppm) ferric chloride, EL at both pH conditions oxidized much faster than SL, as shown in **Figure 2D**. PV of EL reached peak value about 60 meq/kg under both pHs at day 15, and it was only about 25 meq/kg for SL at the two pHs. The effect of pH was not as significant as that shown in lecithin oxidation with 500 μM cupric ion.

Cross-comparing **Figures 2C** and **2D**, it was shown that EL oxidation positively responded to the increasing ferric ion concentration. It is also important that our data show that EL was more sensitive to ferric ion-catalyzed oxidation than that by cupric ion at both pHs.

Many studies have been conducted to examine the relationship between oxidative stability and droplet size of oil-in-water emulsion. A general belief is that small particle size increases surface area, and thus increases the chance of oil droplet being contacted with pro-oxidant transition metal ions, leading to fast lipid oxidation (2, 17). Others reported that particle size of oil droplets did not affect lipid oxidation rate of o/w emulsion (18, 19), or decreased particle size improved oxidative stability of various o/w emulsion (20). Our study suggests that particle size did not significantly affect PL oxidation. As shown in column 1 in **Table 3**, all four treatments had a wide range of particle size though they had similar oxidation trend in terms of PV developments. To have a definitive answer of how oil droplet size affects lipid oxidation, a better designed and controlled experiment needs to be

carried out.

Electric charge of emulsion droplets can be an important factor in lipid oxidation (16). For PL emulsifier, PE and PC are zwitterionic, and PI is anionic. Phosphorus group is negatively charged, whereas choline (in PC) and ethanolamine (in PE) groups are positively charged under neutral condition and, therefore, PC and PE do not carry net charge. Since inositol group in PI has no charge, PI under neutral conditions is negatively charged. In o/w emulsion where transition metal ions are positively charged, transition metal ions will migrate toward the oil droplets that have net negatively charged PLs, causing rapid lipid oxidation. Theoretically, SL should oxidize faster than EL under neutral pH conditions, since SL is negatively charged due to its relatively high PI content. However, the fatty acid composition of lecithin is another important factor in determining lipid oxidation. Therefore, the comparison of the oxidative stability of the two lecithins in emulsions is complicated, and experimental data should reflect the effect of the combined factors.

The surface charge of the oil droplets was measured as zeta potential. As shown in **Table 4**, under neutral conditions both EL and SL showed a decrease in zeta potential as transition metal ions were added, and it is expected. EL consists of PE (18.1%) and PC (78.7%), whereas PE and PC are only 61% in total SL, and the other 40% is mainly the anionic types, i.e. PI and PA (not quantified). Theoretically, SL control should have more negative zeta potential than EL control, but the data did not show this. Similar results were also reported by Sørensen et al (21). **Table 4** also shows that acidic pH had significantly increased zeta potentials compared to that at pH 7. The increased zeta potential at low pH may have contributed to the increased oxidation rate when transition metal ions were added.

Effect of pH and Cupric Concentration on TBARS Development of EL and SL. The TBARS method measures the end products of lipid hydroperoxidation decomposition. As shown in **Figure 4**, EL had higher TBARS development over time than SL, and low pH tended to promote

more TBARS generation. As presented in **Table 2**, SL contained much less C20:4 and C22:6 than EL though SL had more linolenic acid. It was reported that TBARS developed from C20:4 was 8 times higher than that from linolenic acid (22). EL reached a peak TBARS value at day 6 with 0.20 and 0.35 nmol/mg for treatments under pH 7 and pH 3, respectively. SL at pH 7 had no obvious peak throughout the storage, but SL at pH 3 formed TBARS much faster and reached the peak value of 0.16 at day 2. The effect of pH may be due to its catalytic activity in breaking the primary products to secondary products.

For treatments with addition of 50 μ M cupric sulfate, EL and SL showed a similar pattern to the controls. The pH effect became more significant for EL where EL at pH 7 had a peak value of 0.2 and at pH 3 it was 0.42. However, SL did not show a difference between two pH levels (**Figure 5A**).

With higher cupric concentration of 500 μ M, as seen in **Figure 5B**, TBARS of EL at both pH levels increased to about 0.7 at day 2, and pH effect was no longer significant. Also, SL showed increased TBARS, but the TBARS difference between the two lecithins was further increased under higher ionic concentration. SL at pH 3 reached peak value of TBARS of 0.2, and it was 0.1 at pH 7.

A further cross-examination of **Figures 4, 5A, and 5B** helps to understand the effect of cupric concentration on TBARS change of each treatment. For EL in emulsions under both pHs, addition of 50 μ M cupric moved the peak values from day 6 to day 2 although its TBARS value was only slightly increased; further increasing cupric sulfate (500 μ M) caused much faster formation of TBARS during the first 2 days. A similar phenomenon was also observed for SL in emulsions at pH 7. However, adding a large amount of cupric ion did not change SL TBARS as much as in EL under acidic pH.

Effect of pH and Ferric Concentration on TBARS Development of EL and SL. For treatments with 50 μ M ferric chloride addition, EL at pH 3 oxidized considerably faster than that at pH 7 at day 6, but low pH did not accelerate the formation of TBARS for SL, as seen in **Figure 5C**. Still, EL had much higher TBARS than SL under both pH conditions. With higher ferric

concentration, 500 μM (**Figure 5D**), EL at pH 3 showed slightly higher TBARS than that at pH 7. And SL at pH 3 also had overall slightly higher TBARS than that at pH 7. Examining **Figures 4, 5C, and 5D** together, it was observed that adding 50 μM ferric chloride into EL emulsion did not change its time to reach maximal TBARS value, which was 6 days. Also, adding 500 μM ferric ion did not cause much increase in TBARS as it was seen in cupric treatments (**Figures 5B and 5D**). This phenomenon was also observed for SL emulsions. This indicated that cupric ion had a more powerful pro-oxidant effect than ferric ion with respect to TBARS development, and EL was more sensitive to cupric ion-catalyzed oxidation, as determined by TBARS.

For all the treatments, it was observed that TBARS decreased after it reached a peak value. The possible reason for this change of concentration of TBARS over time is that malondialdehyde (MDA) was first produced to a maximal level, and it went down when available fatty acid for MDA formation was depleted (23). Because of this reason, if only simply recording TBARS value at the end of certain storage time, one may miss its peak value and give an improper conclusion. A full examination of oxidation which includes both peak value and the time to reach the value presents a better insight for lipid oxidation.

Relationship between Fatty Acid Composition, PL Class Composition and PV and TBARS Formation. As discussed, lipid oxidation is a process initialized from unsaturated fatty acids. Usually it is believed that lipid-containing foods with high content of PUFAs deteriorate much faster. However, depending on the indicators used for measuring oxidative stability, the results can be different. With respect to PV measurement, many researchers have agreed that linoleic and linolenic acids are oxidized much faster than oleate. But for those long-chain PUFAs, such as AA and DHA, they were found to have lower PV than linoleate in aqueous micelles and certain emulsions (22, 24). In our case, because SL contained 56.6% linoleate, whereas EL had only 16.2% linoleate (**Table 2**), it would be assumed that SL had lower oxidative stability than EL. However, this was found not true.

This indicates that there are many other factors, such as PL class composition, interaction between PL and transition metal ions, that all affect oxidative stability of PLs in emulsions.

Factors influencing TBARS are very complicated. This indicator is not only governed by fatty acid profile but also determined by PL class composition. Pikul et al (25, 26) found that PL contributed about 90% of the total TBARS in a chicken meat containing PLs, TAG, and cholesterol ester. And further study on individual PL fraction revealed that PC and PE produced most of TBARS (77% and 90%) but only about 20% was formed from PS and PI. Our study confirmed this result with respect to TBARS difference between EL and SL. For the effect of fatty acid profile on TBARS development, Visioli et al (22) also pointed out that TBARS production was maximal for DHA and AA which were about 8-10 times higher than that for linoleate. EL contains 1.7% DHA and 6.1% AA, and none was found in SL. This could explain why EL samples had always shown much higher TBARS compared to SL.

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Table 1. Tocopherol content in soy lecithin (SL) as measured by HPLC

Tocopherol	SL, mg/100 g	
	Commercial	Acetone washed
alpha	1.989±0.096	0.032±0.002
gamma	10.976±0.255	0.328±0.018
delta	12.513±0.511	0.698±0.013

Note: the value is presented as mean ± S.D. of the replicates.

Table 2. Major phospholipid class and fatty acid composition (%) of soy and egg lecithin^a

Lecithin	PL, %	C16:0	C18:0	C18:1	C18:2	C18:3	C20:4	C22:6	Other
Egg		30.4	15.9	26.4	16.2	nd	6.1	1.7	3.3
PE	18.1	21.2	30.5	21.1	13.7	nd	13.5	nd	nd
PC	78.7	35.1	12.2	32.5	16.9	nd	1.5	nd	1.8
Soy		20.6	4.3	11.4	56.6	7.1	nd	nd	nd
PE	25.7	20.5	6.4	10.0	56.2	6.9	nd	nd	nd
PC	35.3	16.6	3.0	10.9	59.9	6.6	nd	nd	3.0

^aResults were average of two preparations with maximal coefficient variation of determination of 7%. nd: not detectable.

Table 3. Particle size for the different treatments as volume mean diameter (μm)^a

	EL control	ELCu50	ELCu500	ELFe50	ELFe500
pH 7	6.2 \pm 0.02	11.3 \pm 0.02	18.0 \pm 0.02	11.6 \pm 0.01	16.4 \pm 0.05
pH3	53.2 \pm 0.19	16.5 \pm 0.11	118.0 \pm 0.45	29.2 \pm 0.08	18.3 \pm 0.02
	SL control	SLCu50	SLCu500	SLFe50	SLFe500
pH 7	8.9 \pm 0.01	7.8 \pm 0.02	6.4 \pm 0.01	10.7 \pm 1.82	8.1 \pm 0.01
pH3	253.4 \pm 10.40	227.4 \pm 7.27	8.7 \pm 0.01	171.1 \pm 7.50	7.5 \pm 0.02

^aData represents mean \pm S.D. from two assays. $\text{LSD}_{0.05}$, Least significant difference at $P < 0.05$. EL, egg lecithin stabilized emulsion; SL, soy lecithin stabilized emulsion; Cu50 and Cu500, 50 μM and 500 μM cupric sulfate in the aqueous phase; Fe50 and Fe500, 50 μM and 500 μM ferric chloride in the aqueous phase.

Table 4. Zeta potential (mV) of lecithin-stabilized o/w emulsions as affected by addition of various amounts of metal ions^a

	ELcontrol	ELCu50	ELCu500	ELFe50	ELFe500
pH 7	-44.4±5.7	-37.5±0.6	-36.5±0.2	-38.1±2.5	-34.4±2.3
pH3	--	-66.0±1.6	-57.2±3.0	-51.4±3.0	-52.7±0.7
	SLcontrol	SLCu50	SLCu500	SLFe50	SLFe500
pH 7	-39.1±5.3	-33.8±1.5	-33.3±0.4	-32.1±3.9	-30.7±1.0
pH3	--	--	--	-57.5±1.3	-50.8±1.2

^aData represents means of two assay ± S.D. Certain data under acidic pH were missing because the samples were discarded due to instability at the time of sampling. Refer to footnote in **Table 3** for abbreviations.

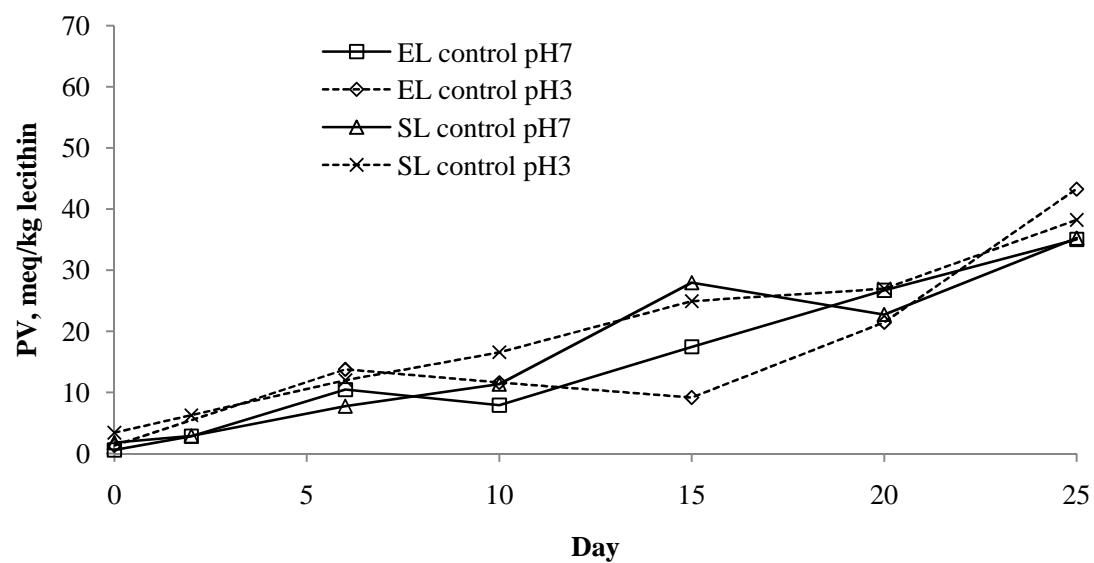


Figure 1. Peroxide value of egg and soy lecithin in emulsion under two pH conditions
Refer to footnote in **Table 3** for abbreviations.

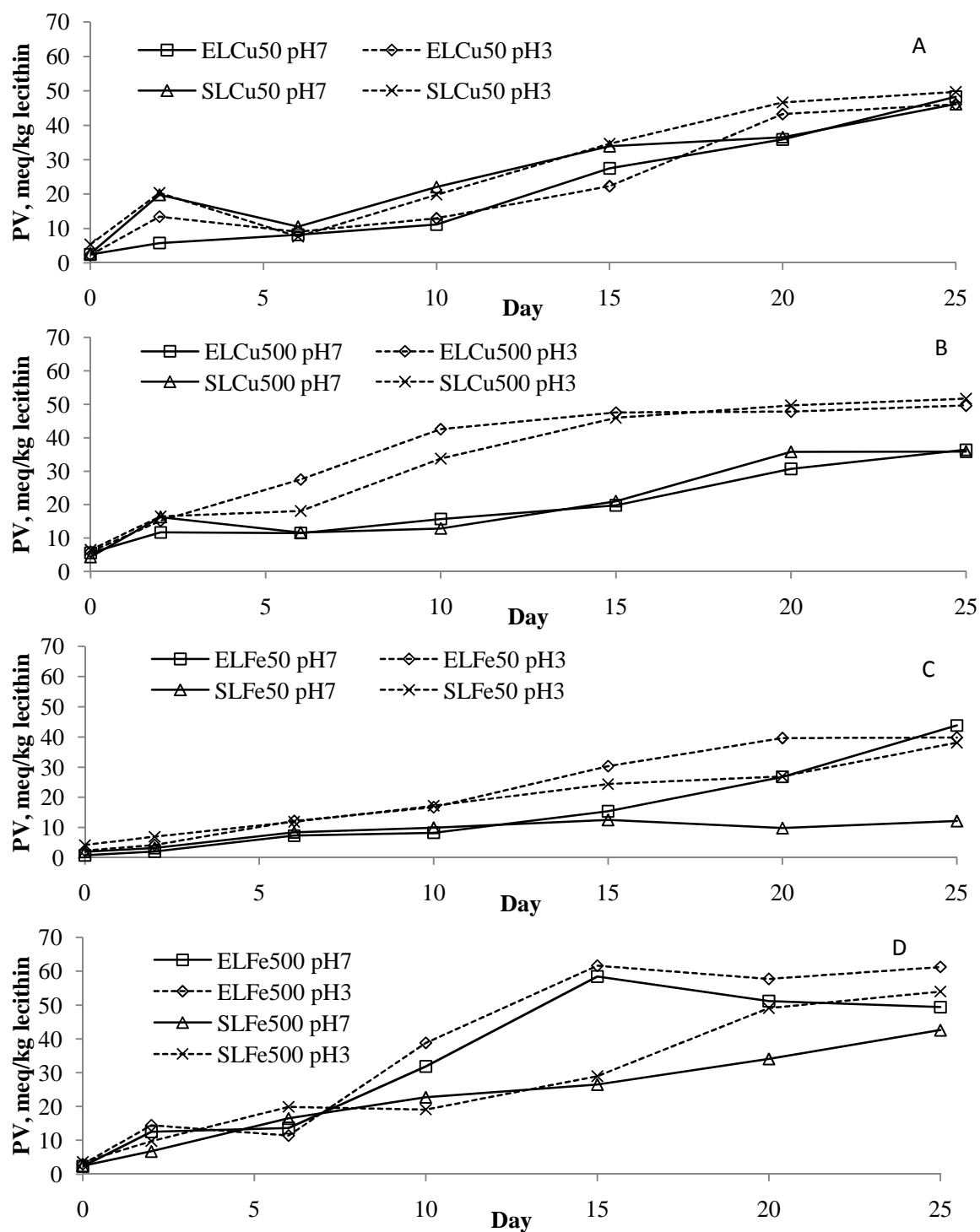


Figure 2. Peroxide value of EL and SL in emulsion under different pH, type and concentration of transition metal ions. Refer to footnote in **Table 3** for abbreviations.

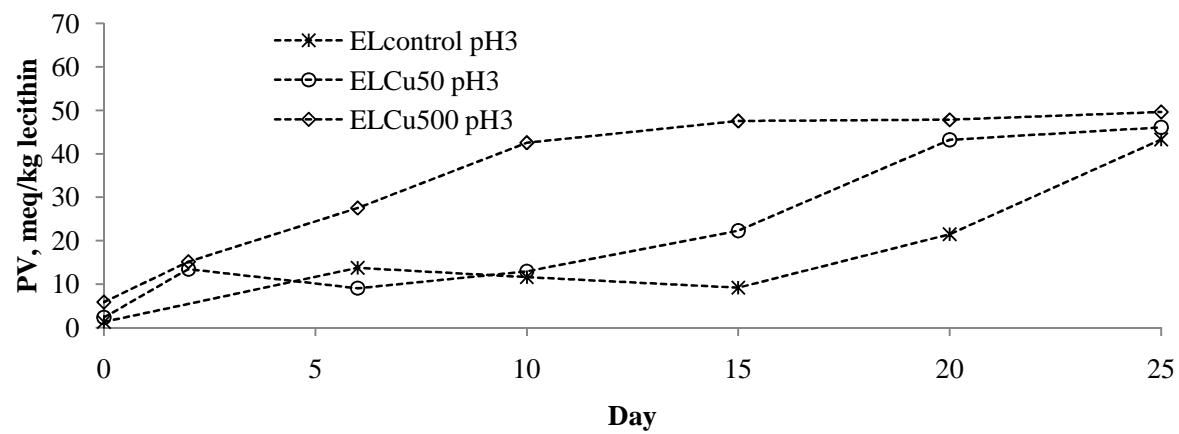


Figure 3. Peroxide value of EL in emulsion as affected by different amount of cupric addition under acidic pH. Refer to footnote in **Table 3** for abbreviations.

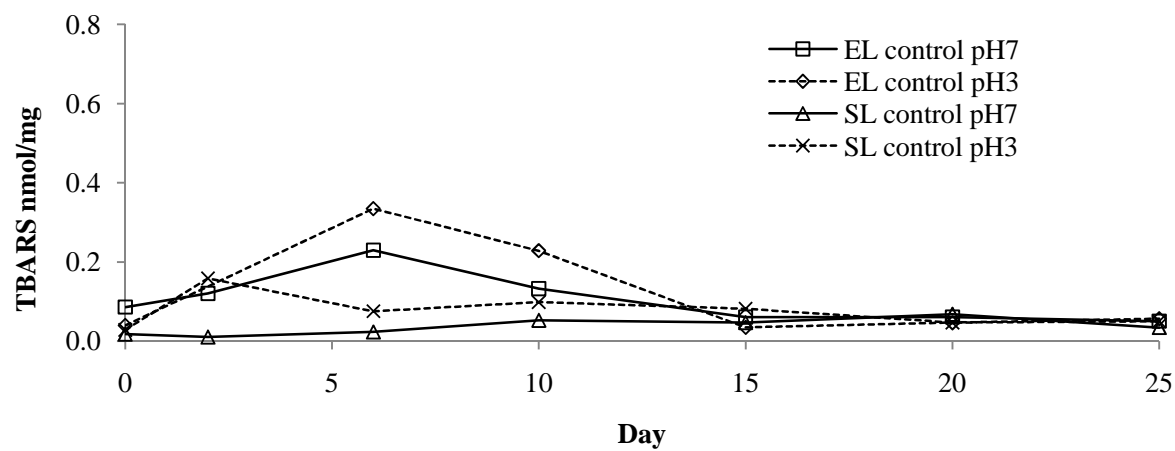


Figure 4. TBARS development of EL and SL in emulsion under different pH conditions. Refer to footnote in **Table 3** for abbreviations.

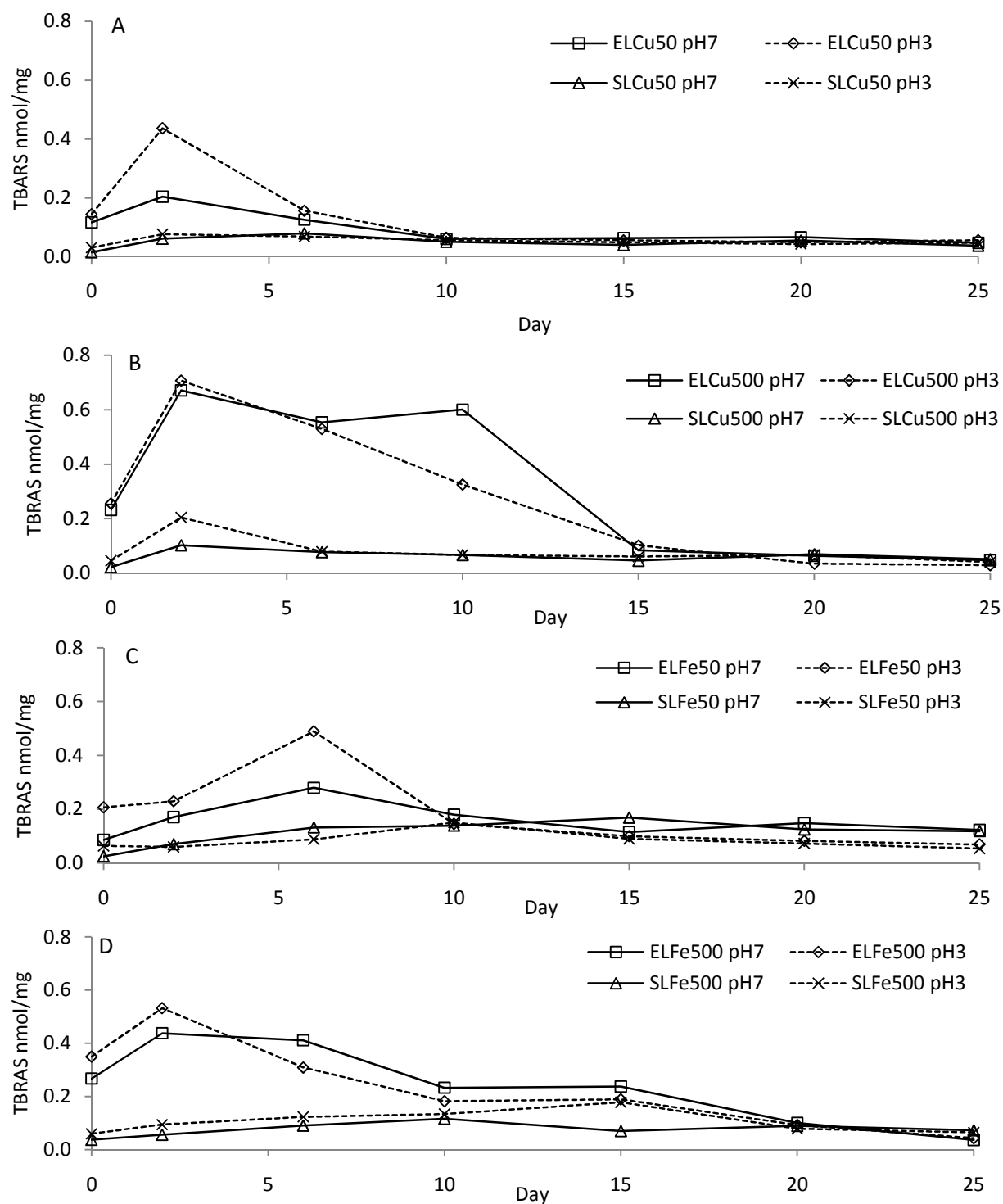


Figure 5. TBARS development of EL and SL in emulsion under different pH, type and concentration of transition metal ions. Refer to footnote in **Table 3** for abbreviations.

CHAPTER 3. THE ROLE OF PLASMALOGEN IN OXIDATIVE STABILITY OF NEUTRAL LIPID AND PHOSPHOLIPIDS

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Guang Wang¹ and Tong Wang^{1, 2}

Abstract

The rule of ethanolamine plasmalogen extracted from bovine brain (BBEP) to maintain oxidative stability of bulk soybean oil and liposomes made with egg phospholipids (PL) was studied. In a purified soybean oil (PSO), the addition of 200 and 1000 ppm BBEP promoted lipid oxidation in a rate of 0.037 and 0.071, whereas soy lecithin (SL) added in the same amount showed a similar trend to the PSO Blank which had an oxidation rate of 0.025. The PSO with BBEP was susceptible to cupric ion-catalyzed oxidation, which was oxidized much faster than the PSO with SL and cupric ion. In commercial soybean oil (CSO) with the presence of tocopherols, SL at 1000 ppm acted synergistically as an antioxidant with the natural tocopherols, but addition of BBEP accelerated lipid oxidation, as evidenced by the oxidative stability index (OSI) test. In the egg PL liposome, the BBEP had a fast breakdown of the lipid hydroperoxides, consequently promoting more thiobarbituric acid-reactive substances (TBARS) formation. The PL oxidation in the presence of copper in the liposome was not affected by the BBEP, which indicates that the hypothesis of ethanolamine plasmalogen (EthPm) chelating cupric ion as the antioxidation mechanism was not supported. The addition of cumene hydroperoxide to the egg PL liposome promoted lipid oxidation as reflected by a fast development of PV and TBARS. However, the result with cumene hydroperoxide failed to differentiate the effect of BBEP and SL and effect of concentration on oxidation. Based on the

¹ Department of Food Science and Human Nutrition.

² Author for correspondence.

observations from this study, we concluded that EthPm is not an antioxidant but rather a pro-oxidant in bulk lipid systems, and it has no significant antioxidant effects for PL oxidation in the liposome.

KEYWORDS: Antioxidant; egg lecithin; lipid oxidation; liposome; plasmalogens; soybean oil; soy lecithin; transition metal ions

Introduction

Plasmalogens are a special class of phospholipids (PL) which share the common structure with other PL except that they have a vinyl ether linkage to the glycerol backbone at the sn-1 position. Plasmalogens are rarely found in plant sources except onion and avocado (1), but they are present in most animal tissues. Ethanolamine plasmalogens (EthPm) are especially rich in brain, testes, and kidney, whereas significant amounts of choline plasmalogens are found in heart and skeletal muscles (2). Several structural and functional properties are associated with plasmalogens in *in vivo* studies, such as increasing membrane fluidity, mediating signal transduction, and relieving free radical-induced cell oxidative stress (3-6).

The antioxidant effect of plasmalogens, especially EthPm, has been studied in both *in vivo* and *in vitro* during the last decade based on the finding that EthPm scavenged reactive oxygen species (ROS) (7, 8). It is reported that one vinyl ether double bond protected four double bonds in arachidonate from oxidation (9), which indicates that EthPm may have certain characteristics of lipid-soluble antioxidants. Zommara et al (10) demonstrated that in a liposome system that contained egg choline PL, the EthPm prevented ferrous- and cupric-promoted lipid oxidation in the presence of hydroperoxide initiators. Similar results were reported by Sindelar et al (11) who found that brain plasmalogens effectively prevented iron-induced lipid peroxidation. The protective role of beef brain lyso-EthPm was claimed by Hahnel et al (8) in the micelles. They also found that the EthPm was

more effective in low density lipoprotein (LDL) particles than in the micelles. In an *in vivo* study, it was found that nerve tissue deficient in plasmalogens was more susceptible to ROS damage, and this deficiency was caused by peroxisomal disorders like Zellweger syndrome; and in the testes, plasmalogens protected spermatocytes from very long chain fatty acid (VLCFA)-induced degeneration and apoptosis (12). However, the molecular mechanism supporting the plasmalogen-mediated prevention of transition metal-induced lipid oxidation is still not well understood. A binding stoichiometry of 1:1 for copper ion: vinyl ether was claimed (8), whereas such chelating was not shown in iron-induced lipid oxidation (11). Many oxidation studies were done with the addition of certain hydroperoxide initiators, especially azo compounds that accelerate the production of hydroperoxide by promoting propagation. However, the use of hydroperoxide initiator is highly questionable because it is an artificial system that is not found in either food or biological systems (13).

While some researchers showed that EthPm functioned as an antioxidant, others showed different results based on the fact that the vinyl ether bond is highly sensitive to oxidative attack (5, 14, 15). The breakdown of vinyl ether bond was observed following the oxygen consumption, and the degradation of plasmalogen was faster in the presence of polyunsaturated fatty acids (PUFA). Rancidity development in meat might also be attributed to the instability of plasmalogens (16). A class of decomposed plasmalogen epoxidation products, α -hydroxyaldehydes, was confirmed, and their high reactivity also raised doubts on the protective effect of EthPm in biological systems (17). Felde and Spiteller (18) also report the α -hydroxyaldehydes as oxidation products of plasmalogen in lipoprotein systems. Another study conducted using plasmalogen-deficient fibroblasts did not show a major role of plasmalogen in protecting cells against ROS as proposed by Zoeller et al (19). Kehrer and Biswal (20) reported a deleterious effect of the Schiff base adducts derived from the aldehydes on

the neural cell membrane. The Schiff base adducts were verified by using GC-MS after the EthPm-containing rat brain homogenates were oxidized under UV and Fe^{2+} /ascorbate (21).

In the present study, we evaluated the antioxidant properties of a plasmalogen using classical lipid oxidation methods, e.g., bulk oil oxidation as indicated by peroxide value (PV) and oxidative stability index (OSI). Oxidative stability of purified soybean oil was compared with that of commercial soybean oil in the presence of BBEP and soy lecithin (SL) in order to determine the possible antioxidant synergy between tocopherols and BBEP. A pure liposome model containing only egg PL (EPL) and water was also used to simulate cell membranes. We were interested in the chelating mechanism as proposed by Hahnel et al (8), so cupric ion was added to both models to verify the proposed chelating mechanism.

Materials and Methods

Materials and Reagents. As a source of EthPm, beef brain was taken from two 21-month-old cows, which were slaughtered in the Meat Lab at Iowa State University. Egg phospholipids (EPL) PL-100M was purchased from Q.P. Corporation (Tokyo, Japan) with a claimed purity of above 99%. Soy phospholipids (SL) was purchased from Sigma-Aldrich (St. Louis, MO). Standard egg phosphatidylethanolamine (eggPE), lyso-eggPE, phosphatidylcholine (soyPC), lyso-soyPC, and phosphatidylinositol (soyPI) were purchased from Avanti Polar Lipid (Alabaster, AL). Soybean oil (Crisco, J.M. Smucker Company, Orrville, OH) was purchased from a local grocery store. All other chemicals were purchased from either Fisher Scientific or Sigma unless otherwise noted.

PE Plasmalogen Extraction. Fresh beef brain tissue was first ground in a Smart Power blender (Cuisinart, E Windsor, NJ) by adding small portion of water. The resulting homogenates were then dehydrated by adding pure ethyl alcohol with manual stirring. The total phospholipids were extracted by using 100% ethyl alcohol and then hexanes as described by Polacios and Wang (22) with slight modification. The polar lipid fraction was fractionated by liquid partitioning, and the polar lipid

fraction was subjected to an acetone wash to remove neutral lipids. The crude polar lipid was further subjected to Folch wash (23) to obtain the pure PL fraction. A further separation of PE fraction was achieved by the thin layer chromatography (TLC). An aliquot of the resulting PL, about 20 mg, was loaded to a 2000 μ m Uniplate silica G thin-layer-plate (Whatman, Piscataway, NJ), which was developed with chloroform: methanol:water (25:10:1, v/v/v). The PE band was scraped into a centrifuge tube and lipid was extracted twice by following the Bligh-Dyer procedure (24). The resulting PE fraction was collected, weighed, and verified by TLC as compared with standard eggPE, soyPC, soyPI, and lyso-soyPC. The final PE fraction (containing PE and EthPm) was dissolved in chloroform: methanol (1:1, v/v) and designated as BBEP. The existence of EthPm was verified by base hydrolysis and the resulting lyso-EthPm was verified by the same TLC method as compared with standard lyso-eggPE.

³¹P NMR Determination of PL in BBEP. Triphenyl phosphate (TPP) was used as an internal standard for the quantification of PL in the BBEP. Purified BBEP (~50 mg) and TPP (~10 mg) were dissolved in chloroform-d (1 mL), methanol (1 mL) and 0.2 N cesium-EDTA solution (1 mL, pH 8.5). After vigorous shaking, the sample was centrifuged, and the lower phase was transferred to a NMR tube (5 mm). The NMR spectra were obtained from a Bruker VXR-400 spectrometer, and it was operated at 400 MHz. Samples were analyzed with the inverse gated decoupling. The NMR spectroscopic scan conditions were as follows: pulse width, 22 μ sec; sweep width, 9718 Hz; acquisition time, 1.2 sec; relaxation delay, 10 sec; number of scans, 256. The chemical shifts were reported relative to TPP (δ -17.8). The data processing was completed using MestReNova software (Santiago de Compostela, Spain).

Fatty Acid Profile of PL. PL fatty acid profile was determined using a Hewlett-Packard model 5890 series II gas chromatograph (GC) with a flame ionizer detector. Ten mgs of PL were first dissolved in 4 mL of sodium methoxide (1 M in methanol). The reaction was conducted for 30 min

under ambient temperature, and was stopped by adding a few drops of water. Fatty acid methyl esters (FAME) were extracted by using hexanes and applied to the GC for analysis. The conditions used for GC analysis were as follows: injection temperature, 230 °C; detector temperature, 230 °C; and the oven temperature was programmed from 110 to 220 °C with a heating rate of 10 °C/minute. The column was a Supelco SP-2330 (Bellefonte, PA) capillary column, 15 m (length) \times 0.25 mm (i.d.) \times 0.2 μ m (film thickness).

Effect of BBEP and Cupric ion on Oxidation of Purified Soybean Oil (PSO). Commercial soybean oil was purified by subjecting it to an activated alumina column (oil to alumina ratio was 1:1) and eluted with distilled petroleum ether to remove tocopherols and other polar impurities following the method from Jensen et al (25). All glassware was soaked in 10% nitric acid and washed with deionized water prior to use. Five grams of PSO were then weighed into 250 mL Erlenmeyer flasks with addition of different amounts of cupric sulfate and BBEP or SL. The treatment arrangement is shown in **Table 1**. Samples were stored in a dark, forced-air oven at 32 °C. The oxidation experiment was conducted for 160 h with a sampling interval of about 12 h. At each sampling time, a drop of 2% butylated hydroxytoluene (BHT) in ethanol was added into the storage vial. All samples were sealed with nitrogen and stored in a -20 °C freezer until analysis. Cupric ion-treated samples were prepared in duplicates.

Effect of BBEP and Cupric ion on Oxidation of Commercial Soybean Oil (CSO). All treatments were prepared in the same manner as that for the purified soybean oil but soybean oil was not treated and used as-is in order to examine the effect of tocopherols on the lipid oxidation with the BBEP, SL, and cupric ion additions. Oxidative stability was measured as an oxidative stability index (OSI, in h) using the ADM Oxidative Stability Instrument (Omnion, Rockland, MA) at 100 °C, according to AOCS Official Method Ca 5a-40 (26). All treatments were in duplicates.

Effect of BBEP and Cupric ion on Oxidation of EPL in Two Liposome Systems. The method to prepare liposome was modified from Zommara et al (10). Briefly, EPL PL-100M was first dissolved in a small quantity of chloroform: methanol (1:1). The solution was transferred to a round bottom flask and a thin film was formed, and then solvent was rotary evaporated. Residual solvent was further removed using a vacuum oven at ambient temperature overnight. The dried lipid film was then dispersed into a solution containing 0.1M tris-HCl buffer and 0.1M sodium chloride at pH 7.4, and the dispersion was vortexed to facilitate lipid dispersion. Then, BBEP (1000 ppm, based on EPL weight) and cupric sulfate (8 ppm, based on EPL weight) were added to the above dispersion. The molar ratio of BBEP to cupric ion was 10:1. The resulting dispersion was sonicated for 5 min with 30 s pause for every 1 min sonication in an ice-water bath by using a Misonix XL sonicator (Farmingdale, NY) with a power setting at 9. The final concentrations were 25 mg/mL, 25 μ g/mL, and 0.2 μ g/mL for EPL, BBEP, and cupric ion, respectively. The size distribution of liposome particles (without adding BBEP and SL) was measured using a Hydro 2000 MU laser scattering particle size analyzer (Malvern, U.K) by following a method from Wang and Wang (27). The oxidation of such liposome was conducted in a dark, forced-air oven at 60 °C for 2 weeks with periodical samplings. Lipid oxidation was terminated by adding a drop of reagents containing BHT and ethylenediaminetetraacetic acid (EDTA) with approximate concentrations of 1 mM and 10 mM, respectively. All samples were filled with nitrogen and stored in a -20 °C freezer until analysis. All treatments were in duplicates.

In order to verify the effect of hydroperoxide initiator on the oxidative behavior of BBEP and SL in liposome, a second experiment was conducted in a different liposome system where the concentration of EPL was 4.0 mg/mL, and the concentrations of BBEP and SL were still 1000 ppm based on EPL; the molar ratio of cupric ion to BBEP was as 1:10; the concentration of cumene hydroperoxide as a lipid soluble hydroperoxide initiator was 40 μ M in liposome. Because the ratio of

plasmalogen to other PL is found high in brain and testes, another BBEP treatment was included with a concentration of 10%, with 10% of SL as a comparison. The liposome samples for all treatments were prepared in the same manner as mentioned above and were stored in a forced-air conventional oven at 37 °C. Sampling was done every 6 h for a total of 30 h.

Peroxide Value and TBARS Determination. To measure the peroxide value (PV) of oxidized soybean oil (PSO), a ferric thiocyanate colorimetric method was used following a previously established procedure (27). Briefly, previously oxidized soybean oil with 19.8 meq/kg of PV, measured by the AOCS Official Method Cd 8-53 (28), was used to establish the standard curve of absorbance versus hydroperoxide (microeqv/kg). PSO samples of 10-100 mg were weighed into 10-mL volumetric flasks, and then the flasks were filled with chloroform: methanol (2:1, v/v). Serial dilution was used for individual samples so that all absorbance values fell within the linear range as specified by the standard curve. Then, 100 μ L of ferrous chloride (0.014 M) and 50 μ L of ammonium thiocyanate (3.75 M) were added to 4 mL of diluted samples and vortexed. The reaction mixture was allowed to stand 20 min, and the absorbance at 500 nm was recorded by using a Genesys 20 spectrophotometer (Cambridge, UK). The PV of EPL liposome was measured in the same manner. For these samples, an oxidized EPL (PV of 29.1 meq/kg) was used to establish the standard curve. Equivalent amount of water as in the liposome was added to the PL standards so that any interference in absorption was controlled.

The method used for determining 2-thiobarbituric acid-reactive substances (TBARS) was described in Wang and Wang's procedure (27), and quantification of TBARS was conducted at 532 nm using the same spectrophotometer as that for PV measurement.

Statistical Analysis. All experiments were conducted with duplicate treatments unless otherwise noted. Data analyses were done by using SAS program (version 9.1, SAS Institute Inc.,

Cary, NC). One-way Analysis of Variance (ANOVA) was used for mean comparisons, and Fisher's least significant differences were calculated at $P < 0.05$ ($LSD_{0.05}$).

Results and Discussion

Chemical composition of BBEP. BBEP was a mixture of EthPm and PE, among which EthPm was 78.2% (mole %), and PE was 17.3%, as quantified by ^{31}P -NMR. With respect to fatty acid profile, EthPm is a rich source of arachidonic acid (AA, 20:4, ω -6, 7%) and docosahexaenoic acid (DHA, 22:6, ω -3, 11%) (**Table 2**). In the plasmalogen molecule, AA and DHA have a tendency to attach at the sn-2 position, which is usually considered to be less susceptible to oxidation than that on the outer position. A lipid oxidation study with individual FFA micelles (29) showed that linoleate produced much more lipid peroxide and conjugated dienes but much less TBARS than DHA and AA. Both the vinyl ether linkage and FA composition make EthPm significantly different from SL which had undetectable AA and DHA and no plasmalogens.

Effect of BBEP on Oxidative Stability of PSO. Commercial soybean oil contains mainly C16 and C18 fatty acids, with 52-55% linoleic acid being the major acid, 8% linolenic acid, and 22-25% oleic acid (30). The ratio of oxidation rate was reported as about 1:12:25 in the order of oleic, linoleic, and linolenic acids in a free radical autoxidation system (31). Therefore, the soybean oil after removing the tocopherols is a good model for studying antioxidant effect because of its richness in double bonds. Our previous test confirmed that at least 99% of the naturally occurring tocopherols were removed by purification with an activated alumina (32).

The oxidation development of PSO, expressed as PV versus time, is shown in **Figure 1**. The relationships between PV and time for all treatments are fitted with exponential regressions, shown as the smooth curves in the Figure. The coefficients of determination (R^2) are present in **Table 1**.

All treatments generally fell into three groups based on PV development and PL class. The first group contains only PSO (Blank or control) and PSO with cupric ion. It was unexpected that the sample containing cupric ion showed low PV development during the storage at 32 °C as for the Blank. The low temperature and the lack of initiators (transition metal ions, peroxides, etc) might have prevented PSO from starting oxidation. However, even after adding cupric ion, PSO still showed no change in PV development as compared with the Blank.

The second group of curves (**Figure 1**) contains SL treatments. Two treatments with 200 and 1000 ppm of SL (SL200 and SL1000) but without copper showed almost the same PV development as the Blank. This indicates that addition of SL had no obvious pro- or antioxidant effect on PSO. However, when 8 ppm of cupric ion was added to these two treatments (SL200/Cu and SL1000/Cu), the increase in oxidation rate was observed. Cupric ion accelerated lipid oxidation in the presence of SL, and SL acted as pro-oxidant because SL1000/Cu was oxidized much faster than that of SL200/Cu. SL may have helped the dispersion of cupric ion in the oil system, so the pro-oxidant effect of cupric ion was observed.

The third group is BBEP treatments with and without cupric ion addition. As 200 ppm of BBEP was added to PSO (BBEP200), the oxidation rate of PSO was increased compared with the first two groups. Based on the fact that SL200 and SL1000 did not promote oxidation of bulk oil but BBEP did, it suggests that BBEP alone acted as a pro-oxidant. When 1000 ppm of BBEP was added (BBEP1000), the oxidation was even more significantly accelerated, validating its pro-oxidant effect. When 8 ppm of cupric ion was added to BBEP treatments (BBEP200/Cu, BBEP1000/Cu), the “lag” or initiation phase of the oxidation disappeared. However, the effect of cupric ion on hydroperoxide propagation (the rate of increase) seemed to be less dramatic than the dose effect of BBEP. This may indicate the main effect of cupric ion in the presence of plasmalogen was to accelerate the initiation of lipid oxidation. **Figure 1** also shows that the cupric treatments with BBEP had much higher initial PV

values than the rest of the treatments. These data clearly demonstrate that lipid oxidation in the presence of EthPm or BBEP was much more susceptible to cupric ion. Therefore, these results failed to support the claim of protective effect of EthPm against transition metal ions-induced lipid oxidation.

Another parameter, oxidation rate derived from data in **Figure 1**, is also shown in **Table 1** for a quantitative comparison. The oxidation rate was defined as the slope of $\ln(PV)$ versus time (in h). It was found that generally SL200, SL200/Cu, and SL1000 had a similar oxidation rate to that of Blank which was 0.025, whereas oxidation rate for BBEP200 (0.037) was almost equal to that of SL1000/Cu. And oxidation rate for BBEP1000 almost doubled from that of BBEP200 and reached 0.071. The value for treatment BBEP200/Cu and BBEP1000/Cu might not reflect the true oxidation process because both treatments oxidized much faster than others, and the initiation stage was lost. So the curve is somewhat atypical.

It should be noted that all cupric ion-treated samples were duplicated, and the others were just single measurement. Because of the size of treatment number and sampling number, we had to use the half of the treatments to show our treatment variation, as shown in **Table 2**, the standard deviation of the oxidation rate. For these duplicates, the reproducibility in general is acceptable (about 12% in average coefficient of variation)

Effect of BBEP on Oxidative Stability of Commercial Soybean Oil (CSO). Commercial soybean oil contains significant amounts of tocopherols (about 1000 ppm) (33), and a synergistic effect was reported between tocopherols and phospholipids contributing to lipid oxidative stability (34, 35). As shown in **Figure 2**, the OSI of CSO Blank (14.1 h) was the same as that with cupric ion-treated CSO (13.6 h), and this observation was the same as that in the purified soybean oil. When 1000 ppm of SL was added to CSO, a significant increase in OSI (16.2 h) was observed in comparison to the Blank, and this is different from the oxidation of purified soybean oil. This

provides a confirmation of the moderate synergistic effect with tocopherols, as reported by other researchers (36). In comparison to SL and CSO Blank, the addition of BBEP at the two concentrations (200 and 1000 ppm) significantly reduced OSI, and this reduction was dose dependent (10.8 h at 200 ppm and 7.9 h at 1000 ppm). When cupric ion was added to the corresponding treatments, both BBEP and SL treatments showed significant reduction in OSI compared with all non-cupric treatments. Furthermore, 1000 ppm of BBEP with cupric ion showed more significant reduction in OSI than its SL counterpart at 1000 ppm. This demonstrates that BBEP failed to chelate cupric ion, and it acted as a pro-oxidant in bulk commercial oil system. All the observations in the commercial soybean oil were consistent with the ones obtained from purified soybean oil with respect to BBEP performance. Regarding the antioxidant synergy of tocopherols and PLs, Bandarra et al (34) proposed that Maillard reaction is possibly involved. We have no reason to believe that BBEP will not have a similar Maillard reaction as PL, but the strong pro-oxidant effect of BBEP might have masked the possible synergistic effect due to the Maillard mechanism.

Effect of BBEP on Oxidative Stability of EPL Liposome in the Absence of Cumene Hydroperoxide. The prepared liposome had a pH of 7.4 and the particle size (volume moment mean of the particle, D[4,3]) of about 20 μm with three size distribution peaks centering at 0.5, 8.0, and 50.0 μm . It was assumed that adding small amounts of BBEP or SL would not dramatically change the size distribution. The liposome appeared milky, and no visually detectable separation was found when it was stored at 60 °C. However, spectrophotometric examination indicated a daily increase in turbidity at 410 nm, which was related to liposome instability. All samples were therefore re-vortexed at each sampling time to ensure homogeneity for sampling.

PV developments for these liposome samples are shown in **Figure 3**. The oxidative behavior of lipid in an aqueous environment is usually more complicated than it is in bulk system because many external factors may significantly influence lipid oxidation, such as impurities, particle size, pH,

and net charge of particles (37,38), in addition to the physicochemical properties of the lipids themselves. Some large variations in PV measurement could possibly be due to hydroperoxide instability in this environment. Such variation should not be due to sampling error because no such significant variation was observed for TBARS measurements, which was done on the same samples. The complexity of the liposome aqueous system might also be responsible for the absence of the classic oxidation curve as seen in bulk oil. Similar PL oxidation curve was also reported by Palacios and Wang (22).

The four treatments can clearly be divided into two groups based in the presence of cupric ion ($LSD_{0.05} = 13.1$). The egg PL liposome with 8 ppm cupric ion (Free Copper) showed no difference as compared to the one with BBEP (1000 ppm) and cupric ion (8 ppm) (BBEP/Cu). PV of both Free Copper and BBEP/Cu had a maximal value (approximately 15 meq/kg) at day 3, and thereafter, they tended to stabilize with the extended storage at 60 °C. This indicates that BBEP did not promote PV accumulation in cupric ion-induced lipid oxidation. In the second group, both Blank and BBEP showed generally higher PV values than that of the first group containing cupric ion. The PV of BBEP seemed to reach a lower peak value earlier than that of the Blank, even though no statistical significance could be shown. An apparent peroxide breakdown was observed for both BBEP and the Blank at relatively lower PV (50 meq/kg) than that typically observed in bulk lipid systems (~400 meq/kg) (22). These data indicate that the addition of BBEP to EPL promoted faster hydroperoxide breakdown as compared to the Blank. The reason why adding copper caused significant reduced PV development is not quite clear just by examining this figure alone. Transition metal ions are known to be good catalysts for the breakdown of hydroperoxides. Therefore, further analysis was conducted to examine products of lipid hydroperoxide breakdown by using the TBARS method.

As shown in **Figure 4**, the TBARS development of the four treatments fell into two groups as well ($LSD_{0.05} = 0.13$) but with an opposite trend as seen in PV development. Free Copper and

BBEP/Cu samples had a significant elevation in TBARS compared to the other two treatments. Combining the TBARS with PV, it is clear that the cupric ion promoted the breakdown of EPL hydroperoxide in the liposome system, therefore, causing less peroxide accumulation. The fact that BBEP did not slow down the breakdown of peroxide in the presence of copper also indicates that BBEP did not chelate copper nor act as an antioxidant. The molar ratio of cupric ion to BBEP in liposome was 1:10 when copper was added at 8 ppm, and theoretically cupric ion should be fully chelated according to the 1:1 chelating mechanism. In comparison to the blank, BBEP alone also promoted the breakdown of peroxide, as evidenced by its higher peak value than that of Blank at day 3. TBARS measures malondialdehyde, formed from the breakdown of hydroperoxides of fatty acids with three or more double bonds. It was reported that the oxidation of AA and DHA produces maximal TBARS, and they were 8-10 times higher than linoleate in a model study (29). This may partially explain why BBEP had a slightly higher peak value than Blank, if the effect of vinyl ether bond is not considered. However, the amount of BBEP used was quite low in comparison with the egg PL base in liposome, so BBEP may have truly accelerated PL oxidation.

As an overall result from PV and TBARS developments, the lipid hydroperoxides in liposome were easily decomposed in the presence of cupric ion. In addition, plasmalogen failed to prevent cupric ion-induced PL oxidation. Plasmalogens also tended to be a pro-oxidant compared to Blank without cupric ion.

Effect of BBEP on Oxidative Stability of EPL Liposome in the Presence of Cumene Hydroperoxide. Cumene hydroperoxide is a fat-soluble oxidizing agent and has been used to accelerate lipid oxidation in other studies (10). As shown in **Figure 5**, 40 μM of cumene peroxide greatly increased lipid oxidation in two aspects: first, the overall starting PV values for all treatments were much higher compared to the results of without cumene as shown in **Figure 3**. Secondly, PV development was much faster, and the values were much higher at lower temperature (37 °C) for

shorter time than those treatments without cumene (60 °C). **Figure 5** also shows that all treatments with cupric ion addition had much higher PVs than those of non-cupric ion treatments. This observation is different from what was found in the absence of cumene hydroperoxide. Lower temperature (37 °C) might have delayed the decomposition of hydroperoxide, whereas cupric ion may have accelerated the release of free radical from cumene hydroperoxide, therefore, greatly accelerating lipid oxidation.

Different from the experiment conducted at 60 °C without cumene hydroperoxide, 1000 ppm of SL were also added to this experiment. It was found that it was the presence of cupric ion that mainly contributed to the higher PV development, and types of phospholipids did not. Data in **Figure 6** also indicate that BBEP or SL neither acted as a pro-oxidant nor as an antioxidant in the cumene hydroperoxide-induced oxidation system. For the other three treatments with 10% addition of SL, BBEP, and EPL to liposome, no significant differences were found among the PL types either. And all three treatments showed similar trends to those of 1000 ppm of treatments (data not shown).

TBARS development for those cumene added treatments is presented in **Figure 6**. The same trend as in **Figure 4** was found except that the addition of cumene peroxide accelerated the formation of TBARS, and cupric ion was still the dominant factor contributing to the higher TBARS. The type of PL (SL and BBEP) had no impacts on TBARS development. The strong oxidizing effect of cumene hydroperoxide might have masked any difference between the types of PLs.

Antioxidant or pro-oxidant? Food antioxidants can be categorized into two classes based on their antioxidant mechanisms, e.g., type I and type II. From the literature, plasmalogens were thought to be a primary antioxidant (Type I). Like tocopherols and polyphenols, they scavenge free radicals and prevent free radicals from attacking double bonds. Phenolic compounds are usually good proton donors, and the phenolic ring structures make themselves resonance-stable upon losing the proton radicals. In reality, plasmalogens don't possess such structural characteristics. On the contrary, its

vinyl ether bond is much more sensitive to oxidative attack (14). Our observations in bulk oil confirmed that it was BBEP not PL that accelerated the lipid oxidation. Other studies also described the decomposed products, α -hydroxyaldehydes, from EthPm oxidation being reactive compounds (17, 18). Felde and Spiteller (18) used human serum lipoproteins, which contain significant levels of EthPm, and proposed an EthPm oxidation by lipid peroxy radicals ($\text{LOO}\cdot$) through epoxide-mediated mechanism at the vinyl double bond. Other researchers (21, 39) found that EthPm oxidation mainly produced α -hydroxyaldehydes of 16:0 and 18:0 rather than aldehydes of 16:0 and 18:0 following a Fe^{2+} /ascorbate oxidation by using rat brain homogenates. The highly reactive hydroxyaldehydes further reacted with the amine group in PE or EthPm to produce Schiff base adducts. Further controversy also might arise from the Schiff base adducts because these Schiff base/Maillard reaction products can be either antioxidant as reviewed by Zamora and Hidalgo (40) or be responsible for deleterious effects on cells (21). It is possible that EthPm may act differently *in vivo* from *in vitro* as the environment can be very different.

Other studies (8-10) indicated that EthPm may act as a secondary antioxidant (Type II), such as chelating agent, like EDTA, phosphoric acid, and citric acid. This class of antioxidants usually chelates certain transition metal ion by sterically hindering formation of the metal hydroperoxide complex or precipitating metal ion from the lipid systems (41). Structurally this class of compounds all has bi- or multidentate ligand containing multiple O, P, or N atoms. It is theoretically doubtful that vinyl ether bonds have such chelating property. More study is needed to validate this hypothesis. Furthermore, some of the plasmalogen oxidation studies employed significant level of azo type compound as free radical initiator. Such practice and validity of results may be questionable.

Considering the fact that the cupric ion accelerated the lipid oxidation in the presence of EthPm, we see the oxidation mechanism as: vinyl ether bond of BBEP is first oxidized because of its low activation energy followed by the formation of epoxide or peroxide. Cupric ion further promotes

the breakdown of epoxide and peroxide with the formation of more free radicals that propagate further lipid oxidation.

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Table 1. Effect of BBEP and SL on oxidation of purified soybean oil at 32°C

Treatment	PL type	PL, ppm	Cu²⁺, ppm	Oxidation rate, slope of Ln(PV). h⁻¹	R² for the exponential regression model
Control	N/A	0	0	0.025	0.918
Free Copper	N/A	0	8	0.026±0.0004	0.890
SL200	SL	200	0	0.026	0.886
BBEP200	BBEP	200	0	0.037	0.989
SL1000	SL	1000	0	0.025	0.906
BBEP1000	BBEP	1000	0	0.071	0.939
SL200/Cu	SL	200	8	0.034±0.005	0.944
BBEP200/Cu	BBEP	200	8	0.030±0.002*	0.940
SL1000/Cu	SL	1000	8	0.041±0.002	0.949
BBEP1000/Cu	BBEP	1000	8	0.033±0.010*	0.966

*Oxidation rate for BBEP200/Cu and BBEP1000/Cu only represented oxidation rate after initiation stage because of their fast oxidation. R², coefficient of determination.

Table 2. Fatty acid composition (mol %) of phospholipids from different sources

PL source/FA type	C16:0	C18:0	C18:1	C18:2	C18:3	C20:4	C22:6	Others
BBEP	12	11	31	20	nd	7	11	8
EPL	30	16	26	16	nd	6	2	5
SL	21	4	11	57	7	nd	nd	2

Note: BBEP, beef brain ethanolamine plasmalogen; SL, soy lecithin; EPL, egg phospholipid. Nd. Not detected.

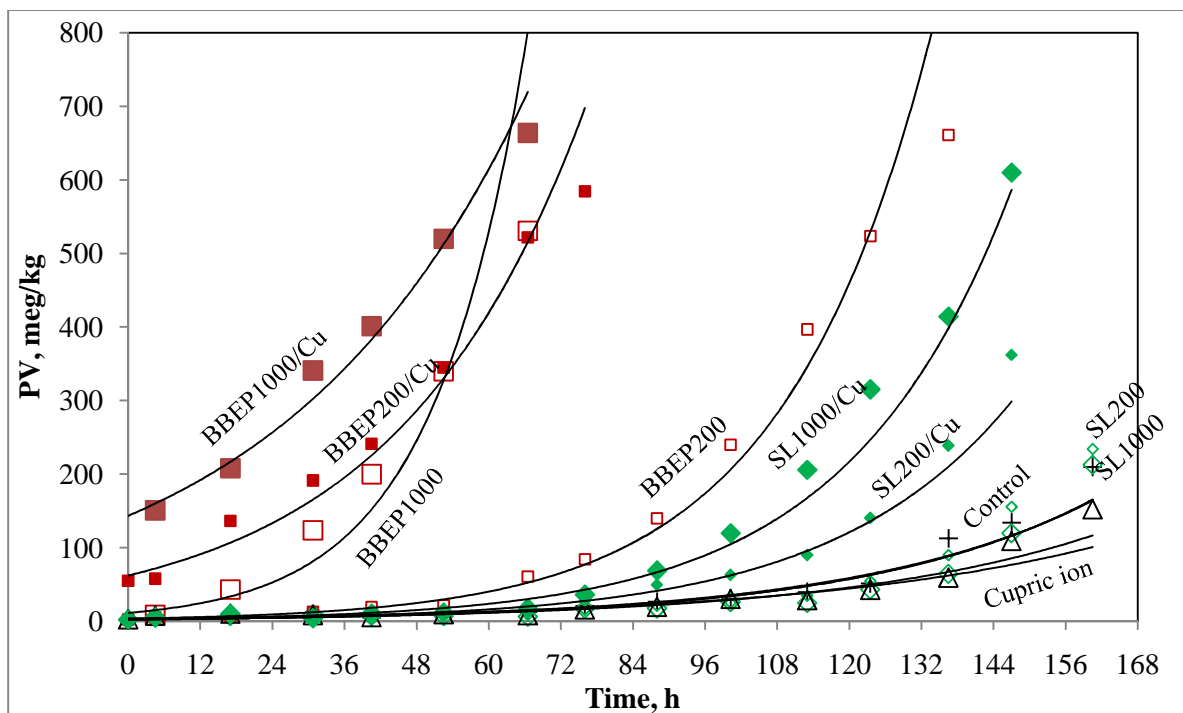


Figure 1. PV development of purified soybean oil at 32 °C in the presence of SL and BBEP fraction (200 and 1000 ppm) and cupric ion (8 ppm). Trendlines are drawn by following exponential regression models. For treatment abbreviations, refer to footnotes of Table 1 and Table 2.

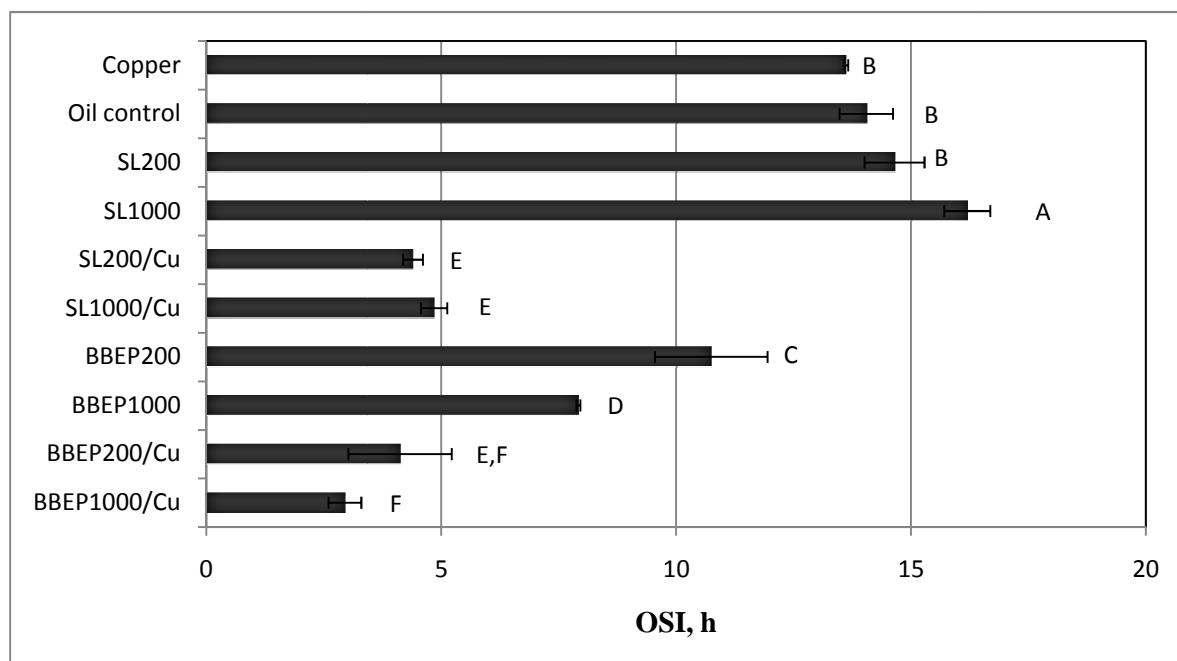


Figure 2. OSI value (time, h) of commercial soybean oil (CSO) at 100 °C in the presence of SL and BBEP fraction (200 and 1000 ppm) and cupric ion (8 ppm). Error bars represent S.D. from duplicate preparations. For treatment abbreviations, refer to footnotes of Table 1 and Table 2. Different letters represent least significant difference at $P < 0.05$.

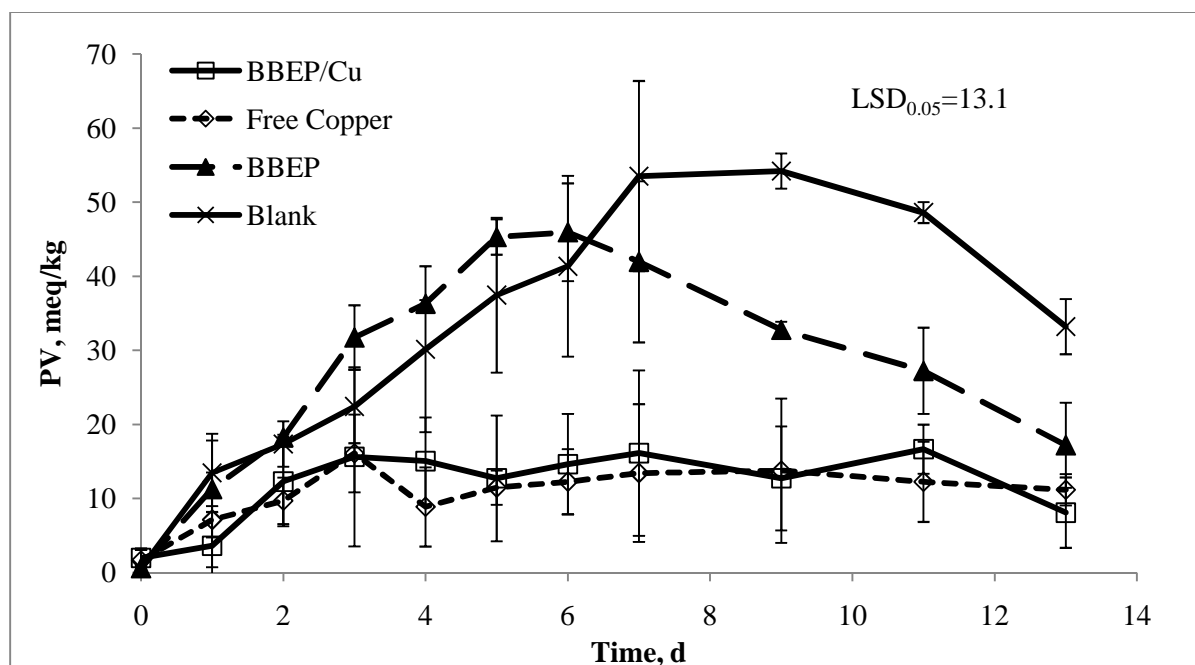


Figure 3. PV development of egg phospholipids in liposome at 60 °C in the presence of BBEP (1000 ppm) and cupric ion (8 ppm). Error bars represent S.D. from duplicate preparations. For treatment abbreviations, refer to footnotes of Table 1 and Table 2.

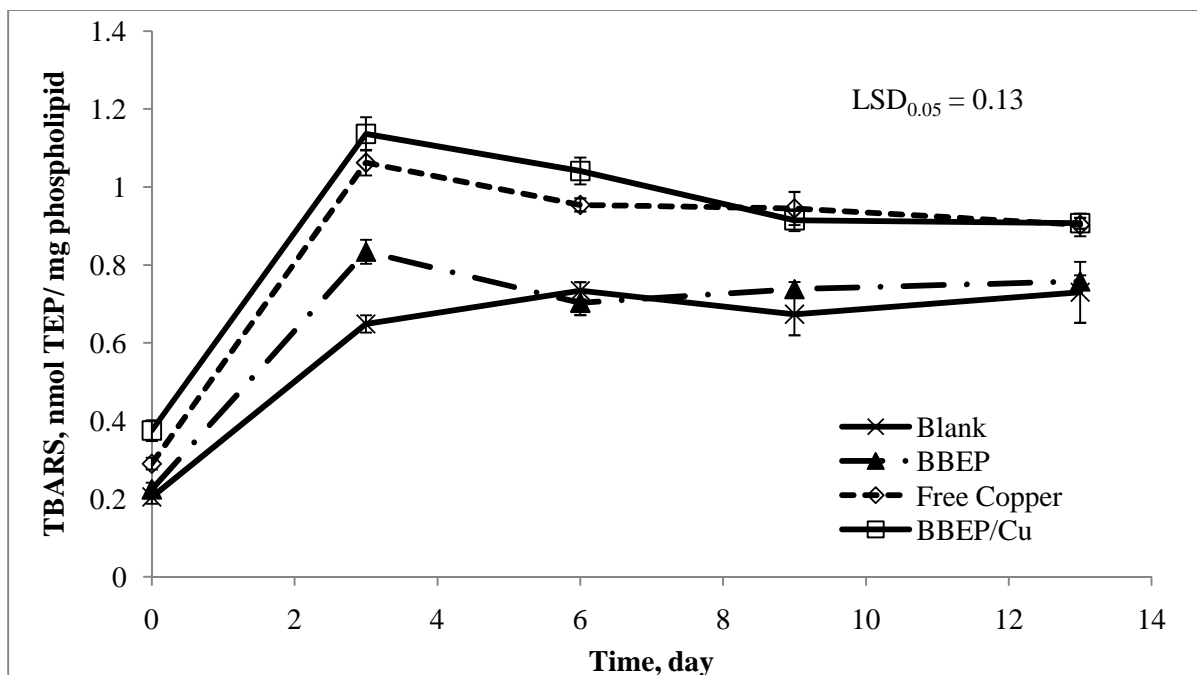


Figure 4. TBARS development of egg PL in liposome system at 60 °C in the presence of 1000 ppm BBEP and 8 ppm cupric ion. Error bars represent S.D. from duplicate preparations. For treatment abbreviations, refer to footnotes of Table 1 and Table 2.

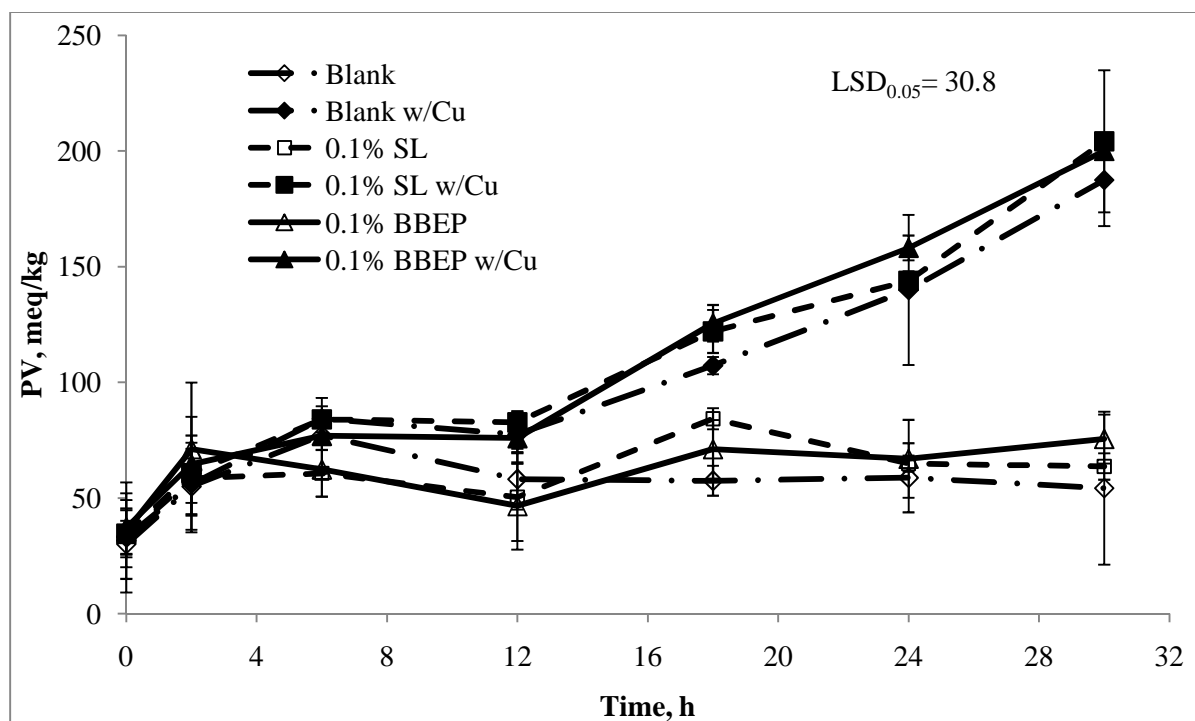


Figure 5. PV development of egg phospholipids in liposome at 37 °C in the presence of cumene hydroperoxide (40 μ M in liposome). Error bars represent S.D. from duplicate preparations. The concentration of SL and BBEP is 1000 ppm based on EPL. For treatment abbreviations, refer to footnotes of Table 1 and Table 2.

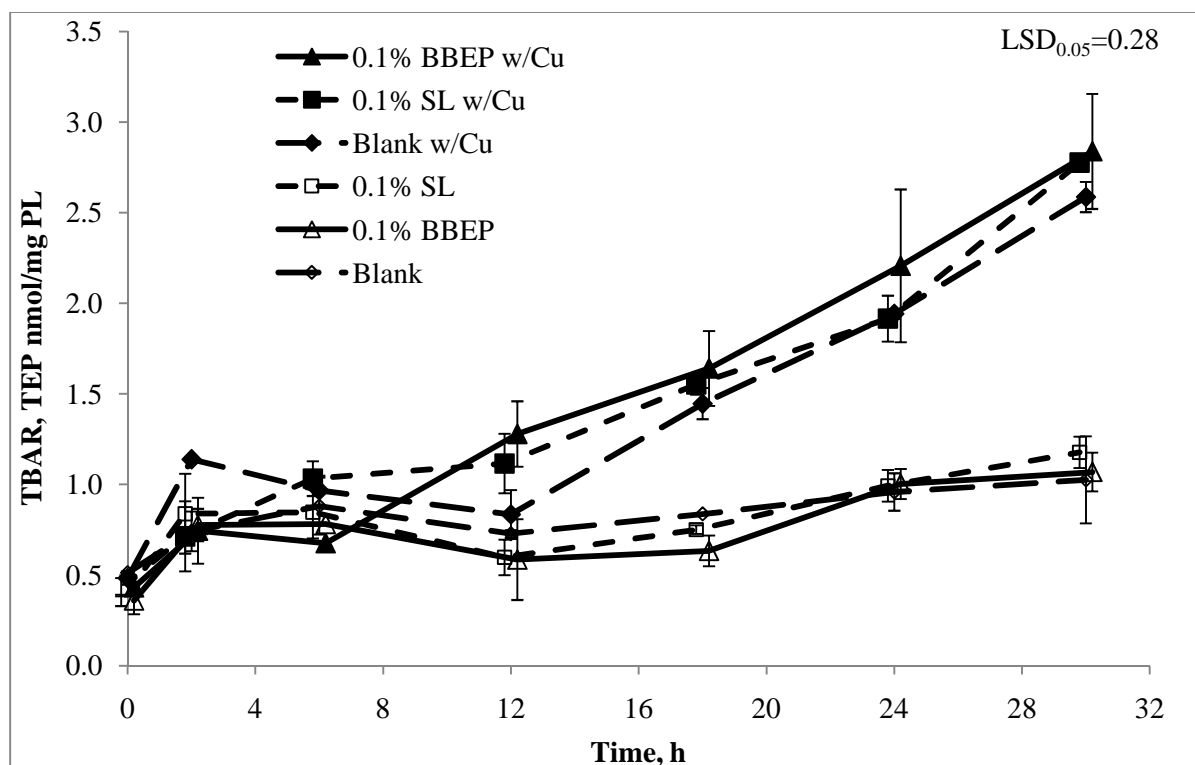


Figure 6. TBARS development of egg phospholipid in liposome at 37 °C in the presence of cumene hydroperoxide (40 μ M in liposome). Error bars represent S.D. from duplicate preparations. The concentration of SL and BBEP is 1000 ppm based on EPL. For treatment abbreviations, refer to footnotes of Table 1 and Table 2.

CHAPTER 4. EFFECTS OF YOLK CONTAMINATION, SHEARING, AND HEATING ON FOAMING PROPERTIES OF FRESH EGG WHITE

A paper adapted from the publication in the *Journal of Food Science*¹

Guang Wang² and Tong Wang^{2,3}

Abstract

A series of experiments were conducted to evaluate the effects of yolk contamination, shearing, and thermal treatment on foaming properties of liquid egg white. Samples obtained from industrial processing were also evaluated. Whipping and sparging methods were both used to assess their effectiveness and sensitivity in evaluating foaming. A concentration as low as 0.022% (as-is basis) of yolk contamination caused significant reductions in foaming capacity and foaming speed. The neutral lipid fraction of egg yolk caused the major detrimental effect on foaming, whereas the phospholipids fraction did not give significant foaming reduction at a concentration as high as 0.1%. High-speed and short-time shearing caused no apparent damage but longer shearing time significantly impaired foaming. Heat-induced foaming change is a function of temperature and holding time. Foaming was significantly reduced at a temperature of 55 °C for 10 min, whereas it did not change up to 3 min at a heating temperature of 62 to 64 °C. Industrial processing steps (pumping, pipe transfer, and storage) did not produce negative effects on foaming of the final products and controlled pasteurization was actually beneficial for good foaming performance. Therefore, yolk contamination of the egg white was the major factor in reducing foaming properties of the white protein.

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² Department of Food Science and Human Nutrition

³ Author for correspondence

Keywords: egg white, foaming, lipid tolerance, processing factors, shearing, thermal treatment, yolk contamination

Introduction

The application of liquid egg white in making meringues and angel food cakes is primarily attributed to its excellent foaming properties. Foam is a colloidal dispersion of air in a liquid phase. A good surface-active protein has to rapidly migrate to interface, unfold readily, and have relatively high visco-elasticity for a stable film (Clarkson and others 1999). Many studies have been conducted to investigate in model systems the function of individual egg white protein fractions and interaction of these proteins on foaming (Cunningham 1976; Yang and Baldwin 1995; Damodaran and others 1998; Relkin and others 1999; Lechevalier and others 2003).

Yolk contamination of white has always been a serious problem in egg processing. It seems impossible to avoid any yolk contamination of egg white even with the advanced egg cracking and breaking technology. Early research found that 1 drop of yolk in 30 mL of egg white was sufficient to reduce the egg white foam volume from 135 to 40 mL (St. John and Flor 1931; Kobayashi and others 1980). Smith (1959) found that the angel food cake failed to foam when neutral lipid triglyceride (equivalent to 0.1% yolk contamination) was added to egg white. But this result was not satisfactory because of its poor reproducibility. Other researchers (Cotterill and Funk 1963; Lomakina and Mikova 2006) also reported a similar result of the effect of neutral lipid. On the other hand, some believed that phospholipids mainly contributed to the impaired foaming either in egg white or other protein systems (Damodaran 1996; Zayas 1997; Alzagat and Alli 2002). Poole (1989) found that whey protein isolate (WPI) with 0.1% lecithin performed much worse than when neutral lipid, such as corn oil, coconut oil, and butter oil was added. In addition, diminished volume of sponge cake was reported as 1%, 2%, or 4% lecithin was added to white (Cunningham 1975). To better understand

whether neutral oils or polar phospholipids are more detrimental in reducing the foaming properties of egg white, more research is needed, and finding a threshold of yolk contamination that will cause change in foaming of egg white is desirable for industrial operation.

Along with the intrinsic protein quality, many environmental factors encountered in egg processing plants may affect egg foaming as reviewed by several researchers (Yang and Baldwin 1995; Lechevalier and others 2005; Lomakina and Mikova 2006). These include mechanical treatments (stirring, blending, pumping transfer) and thermal treatments (heating, cooling, and pasteurization). Egg white contains several proteins including ovalbumin (54%), ovotransferrin (12%), ovomucoid (11%), ovomucin (3.5%), lysozyme (3.4%), and other minor protein components (Stadelman and Cotterill 1995). These major proteins all have denaturation temperature (T_d) higher than 65°C except ovotransferrin which has T_d of 61 °C. The most often used thermal process in egg production is pasteurization which normally kills more than 99% of the bacteria in raw egg. U.S. Department of Agriculture (USDA) recommends that pasteurization for liquid egg white should be at temperature higher than 52 °C with proper holding time. Most of the egg manufacturers conduct a high-temperature-short-time (HTST) process which usually uses a temperature of 56.7 °C for 3.5 min. Most researchers agree that impairment occurred when egg white was heated for several minutes above 57° C (Stadelman and Cotterill 1995). A statistical analysis of the effects of industrial processing steps on functional properties of pasteurized liquid egg white was conducted, and the results showed a reduction of 8% in foam stability between the start and the end processing (Lechevalier and others 2005). On the other hand, it was also reported that suitable heat treatments were beneficial to foaming of yolk-contaminated white (Cunningham and Cotterill 1964). Further verification is needed because practical processing parameters may vary under the currently faster and more advanced egg breaking and treatment systems.

Our research objectives were to use 2 foaming methods to evaluate the effect of yolk and

each of the 2 lipid fractions on foaming properties of egg white and to determine how shearing and thermal treatments affect egg white foaming in our model system and in the plant processing.

Materials and Methods

Foaming determination by nitrogen sparging method

A foaming device made by fusing a fritted glass disk with graduated glass cylinder was used in this experiment (Wang and others 2004). Nitrogen gas was sparged at 100 mL/min to make a final foam volume of 300 mL from 100 mL of 0.5% treated egg white protein. Three measurements were made: time to reach final volume of 300 mL, t_f in seconds; volume of liquid sample consumed at t_f , V_{\max} in milliliter; and time used for half of the incorporated liquid to drain back, $t_{1/2}$ in seconds. Three foaming parameters were calculated based on these measurements:

1. Foaming capacity, which was mL of foam formed per mL of N₂ purged, calculated as:

$$F_c = \frac{300 \text{ mL}}{100 \text{ mL} / \text{min} \times t_f \times 1 \text{ min} / 60 \text{ sec}}, \text{ in mL/mL}$$

2. K value, which was used to describe foam stability, calculated as:

$$K = \frac{1}{V_{\max} \times t_{1/2}}, \text{ in mL}^{-1} \text{ s}^{-1}$$

3. V_i , which was the speed of liquid being incorporated into foams:

$$V_i = \frac{V_{\max}}{t_f}, \text{ in mL s}^{-1}$$

All samples were gently and thoroughly mixed for 30 minutes and then tested for foaming properties by this sparging method. All treatments were done in 3 replications and the mean values were reported.

Foaming determination by whipping method

Whipping is the most frequently used method to measure foaming properties of protein solutions in industry. A series of 5% egg white protein solutions with different amounts of yolk and lipid addition was tested by this method. Whipping was done at setting 10 for 90 s with a KitchenAid blender (Ultra Power Standard Mixer, KitchenAid, St. Joseph, Mich., U.S.A.) using 100 mL sample solution. The final volume after whipping and the liquid volume drained from the foam after 30 min were recorded for the calculation of foaming properties. Three foaming parameters were calculated as described by Poole and others (1986):

Foam expansion (FE), % = $100 \times (\text{total volume of foam and liquid} - \text{initial volume of liquid}) / \text{initial volume of liquid}$;

Foam stability (FS), % = $100 \times (\text{foam volume after 30 min} / \text{initial foam volume})$; and

Foam liquid stability (FLS), % = $100 \times (\text{volume of liquid drained from foam after 30 min} / \text{initial volume of liquid})$.

All treatments were performed in duplicates and mean was reported.

Effect of yolk contamination on foaming properties of egg white

Fresh eggs purchased from a local store were stored in a refrigerator for less than 24 h before use. White was carefully separated from yolk using a kitchen filter and chalazas were removed. Residual egg white on yolk was removed by rolling the yolk over on a paper towel. Both egg white and yolk were gently stirred to obtain uniform appearance. To conduct the nitrogen sparging test, different amounts of fresh yolk were mixed with 50 g egg white to have final fresh yolk concentration ranging from 0% to 1.8%. Another set of treatments with yolk samples concentration up to 0.5% was prepared for the whipping test.

Effect of lipid type on foaming properties of egg white

Egg yolk was fractionated into neutral lipid (triacylglycerols, TAG) and phospholipids (PL)

by using an ethanol–hexanes sequential extraction procedure (Palacios and Wang 2005). Neutral lipid with less than 2.5% of PL and PL fractions with purity greater than 95% were obtained. Both TAG and PL were first dispersed into 50 mL of deionized water followed by adding 50 mL of freshly prepared egg white to obtain 5% of egg white protein for the whipping test. PL and TAG additions from 0 to 0.8% of egg white were used.

Shearing treatments

Egg white and yolk were separated as described previously. The egg white was gently stirred to achieve uniform appearance. Samples were stirred for 5, 10, and 60 s in a Cuisinart brand blender (Smart Power blender, Cuisinart, Stamford, Conn., U.S.A.) with 7 speed settings initially. The STIR setting (a rotor speed of 14,000 rpm) was used for this experiment. After stirring, the egg white was divided into 2 containers where whites were diluted with water to final concentrations of 0.5% and 5%. Egg white without experiencing the shearing treatments was used as a control. All samples were gently stirred using magnetic stirrers for 30 min to obtain fully dispersed protein solution. Whipping and nitrogen sparging were done according to the above-mentioned procedures. Treatments were duplicates, and samples for whipping test were duplicated and for the sparging test were triplicates. To verify the effect of longer shearing time on the foaming properties, a 2nd experiment was conducted with shearing time of 1, 3, and 5 min under the identical conditions as for the 1st experiment.

Heating treatments

Fresh egg white was obtained as described previously. Egg white was gently stirred to achieve uniform appearance. Bulk white in a 200 mL beaker was put into water bath with controlled temperature at 55 °C and gently stirred with a mixer (Arthur H. Thomas Co., Pa., U.S.A.) with a speed of 40 rpm. After heated for 10, 30, 60, 90, 120, and 150 min, samples were taken and cooled to ambient temperature. All samples for the foaming test were measured within the same day.

Before the whipping or sparging measurement was conducted, the bulk egg white sample was divided into 2 containers where they were diluted to 5% and 0.5% concentrations. Egg white without heating was used as a control. All samples were gently stirred for 30 min to obtain fully dispersed protein solutions. Whipping and nitrogen sparging were done according to procedures described previously. All tests were based on single factor completely randomized design. To verify the effect of severe heating on foaming properties, a 2nd experiment was done with heating temperature at 65 °C for 1, 2, 3, 4, 5, and 6 min with 40 rpm gentle stirring. Only whipping method was used to characterize foaming properties for this set of treatments. It is worth noting that when heating temperature was higher than 65 °C, coagulation was observed, and the size of coagulates increased with time.

Statistical analysis

All preparations were in duplicates. SAS system (version 9.1.3, SAS Inst. Inc., Cary, N.C., U.S.A.) was used for data analysis. One-way analysis of variance (ANOVA) was used for multiple mean comparison, and Fisher's least significant difference (LSD) values were calculated at $P < 0.05$ for the pair-wise comparison of the means.

Results and Discussion

Effect of yolk contamination on foaming properties of egg white

A series of yolk-contaminated egg white with yolk concentration from 0.022% to 1.8% (fresh yolk in white) were prepared to conduct the nitrogen sparging test. The results are shown in Figure 1. For foaming capacity (F_c), a significant reduction from 1.87 to 1.67 mL/mL was observed even with addition of yolk as low as 0.022 g in 100 g egg white, which suggests that egg white has almost no tolerance toward any yolk contamination with respect to foam volume. Further addition of yolk continued to decrease F_c until yolk contamination approached about 0.03%, at which a relatively

constant value was reached and foam volume only decreased slightly with higher level of yolk addition. For foam stability, a low K value indicates more stable foam. As shown in Figure 1, K value increased sharply with addition of yolk and a significant change was found when yolk contamination was as low as 0.026%, which was 0.000167 per mL s as compared with 0.000114 per mL s of the control. At the point of 0.05% yolk contamination and thereafter, K increased only slightly with a final value of about 0.00002 per mL s at 1.8% yolk contamination. For foaming speed, V_i , followed a similar pattern as F_c . V_i significantly reduced from 0.479 to 0.389 mL/s when only 0.022% yolk was added to the white. This result further confirms that minute yolk contamination can lead to a significant reduction in foaming properties of egg white, especially foaming capacity and foaming speed. The results from the 3 sparging parameters confirmed that there is practically no tolerance for yolk contamination to egg white protein in terms of foaming properties. St. John and Flor (1931) mentioned that 1 drop of yolk in 30 mL egg white caused a reduction in foam volume. One drop of yolk is about 20 to 40 mg in weight. Therefore, the yolk contamination level was actually about 0.07% to 0.14% (w/w) in their study.

The foaming properties as measured by the whipping method are presented in the right side column of Figure 1. As shown, foaming properties of egg white were significantly influenced by the presence of egg yolk, even at a very low concentration (0.04% yolk contamination of the white). There was a steady change in each of the 3 parameters with increasing yolk concentration. Samples with even lower egg yolk concentration (0.01%) were also tested and it was found that foam expansion was reduced by 8% compared to the control.

From the 2 sets of figures, we see that the 2 foaming methods positively correlated with each other. Sparging method is shown to be more sensitive at lower yolk concentration (0% to 0.1%) and whipping method worked well throughout the range tested (0% to 0.5%). A collaborative study also showed that whipping method was effective and related the best to the industrial processes (Phillips

and others 1990). Sparging method was also used by others (Waniska and Kinsella 1979; Baniel and others 1997) but with different designs. Sparging has some advantages over whipping, such as low protein concentration (0.5%) is needed as compared with 5% for whipping method, and foaming is relatively quick.

Egg white foaming is susceptible to yolk contamination as indicated by the effect of minute amount (0.03%) of yolk contamination (Smith 1959). In our experiment, the lowest tested concentration of 0.022% was shown to have a significant impact on foaming by the sparging method. With the whipping method, even lower amounts of yolk (0.01%) contamination had a negative effect on foam expansion. These experiments further verified the effect of yolk contamination on foaming properties of egg white, and provided data to show that even at lower yolk concentration than previously suggested, foaming of white is reduced.

Effect of lipid type on foaming properties of egg white

As stated, both TAG and PL were fractionated from fresh egg yolk and both had purity greater than 95%. Pure egg white was used as a control. The effect of individual TAG and PL addition on foaming properties of egg white is shown in Figure 2. Our preliminary test was conducted under even lower lipid concentration which was from 0% to 0.2%. The result shows that no significant difference was observed between TAG and PL when addition of lipid was up to 0.04% (data not shown). Their difference became significant when lipid addition was more than 0.04%, and foam expansion of TAG-treated samples became significantly lower than that of pure white control as well as lower than the PL-treated samples. However, the difference of foam stability between the 2 lipid treatments was not as significant as that of foam expansion at low lipid concentration. Figure 2 presents the foam properties when up to 0.8% of each TAG and PL was added to fresh egg white, and it shows a clear foaming comparison between TAG- and PL-treated egg white. As shown, as much as 0.05% of TAG addition caused significant reduction in foam expansion as compared to both the

control and PL-treated samples. This is consistent with the findings of our preliminary test. The general trend in Figure 2 reveals that TAG-treated egg white showed much faster damage in all 3 foaming parameters (foam expansion, foam stability, and foam liquid stability) than PL-treated samples. Especially for foam expansion, TAG-treated samples lost almost 100% of their foam expansion as TAG addition was increased to 0.4%, but PL-treated samples still sustained around 85% of foam expansion compared to the fresh egg white. Foam stability and foam liquid stability started to show difference between TAG and PL-treated samples as lipid addition was higher than 0.15%. This experiment clearly suggests that at higher level of lipid addition, TAG-treated egg white is much more damaged than the PL-treated samples.

Fresh egg yolk has about 50% of water content and 31% to 35% of lipid. Within the lipid fraction TAG is about two-thirds and PL is about one third of the total. So the amounts of TAG and PL in 0.4% yolk-contaminated egg white are equivalent to 0.08% TAG and 0.04% PL addition in fresh egg white. At such yolk contamination level, the foaming was greatly damaged as shown in Figure 1, and the damage is convincingly due to TAG, not PL, as shown in Figure 2.

It was also seen from this experiment, as low as 0.02% yolk contamination caused a significant damage to egg white foaming. Such amount of yolk is equivalent to 0.004% TAG and 0.002% PL addition. However, in each TAG and PL foaming experiment, the lowest amounts of TAG and PL needed for significant foaming reduction seemed much higher than these calculated concentrations. So it is possible that the low concentrations of TAG and PLs have certain synergistic effect on foaming reduction.

The detrimental effect of yolk on foaming of egg white essentially arises from protein–lipid interactions at the air–liquid interface. Fresh egg white contains about 10% of protein and no lipid, whereas egg yolk contains not only proteins but also all types of lipids, such as TAG, cholesterol, and PL. Lipids, especially TAG, reduce protein foaming properties by competitive adsorption and

disturbing protein–protein hydrophobic interaction on the interface. TAG is not only much smaller in size than protein but also more hydrophobic than PL and protein. The relative amount of TAG (63%) is also more than that of PL (31%) in yolk lipid (Sikorski and Kolakowska 2003). This TAG contamination prevents the effective protein molecules from forming a strong film. This may explain why TAG rather than PL had more detrimental effect on egg white foaming. Low levels of PL did not contribute to foaming damage as in this study but did damage protein foaming when its addition level was much higher as seen in Figure 2. Similar results were also reported by Cunningham (1975). Some researchers (Sarker and Wilde 1999) proposed that PLs stabilize foam by a different mechanism from that of protein and that these 2 mechanisms are not compatible in a protein-dominated foaming system. Foams formed with proteins are rigid and visco-elastic, whereas lipids tend to form a dense, fluid interfacial layer with reduced interfacial tension. Foam becomes less stable when both polar lipids and proteins are at the interface. The visco-elasticity arising from intermolecular interaction between proteins was reduced by lipid molecules and the mobility of the lipid is compromised by the protein (Sarker and Wilde 1999; Fillery-Travis and others 2000).

Effect of shearing on foaming properties of egg white

Mechanical shearing during plant processing of egg white is encountered in many steps, such as stirring, blending, pumping, and piping transfer. Up to date there is no sufficient information in the literature that quantitatively addresses the relationship between shearing and protein denaturation. In a laboratory study, it is difficult to mimic the real shearing stress as it is encountered in plant processing. In this study, several trials were conducted by treating egg white under different shearing conditions to observe their foaming performance. The trials showed that under the STIR setting of the blender we used, foaming changed by short-time blending treatment. So this setting was selected and its rotor speed was measured as 14000 rpm. The results from sparging test revealed that short-time shearing (less than 1 min as shown in left-side column of Figure 3) did not significantly change

foaming capacity, foaming speed, and foam stability. When longer shearing was applied as seen on the right column in Figure 3, foaming capacity slightly decreased with time. For foam stability K and foaming speed V_i , egg white started to show a significant difference after 1-min shearing compared to the non-shearing control. And no further change was observed thereafter. Based on these observations, we conclude that egg white foaming is tolerant to short-time and high-speed shearing, but the detrimental effect is significant when shearing time is longer than 1 min at the given stirring speed of 14000 rpm. We recognize that this shearing setting is probably much higher than what is encountered in industrial processing.

The foaming properties as measured by the whipping test are presented in Figure 4. Foam expansion was improved slightly by short-time shearing, whereas there was no significant difference in foam stability and foam liquid stability. The results from the longer shearing treatments were consistent with that from the sparging test but the trends were more noticeable. Foam expansion decreased when shearing time was longer than 1 min, and after 3-min shearing both foam stability and foam liquid stability also significantly decreased.

High-speed stirring may partially denature the egg white protein by exposing its interior hydrophobic core, therefore, may show a stronger protein–protein interaction. Over-shearing may cause reduced foaming due to total denaturation of protein, causing protein coagulation and precipitation due to strong intermolecular hydrophobic interaction. Our study quantified the effect of shearing that was suggested by other researchers (Henry and Barbour 1933).

Effect of heating 55 °C on foaming properties

In our laboratory trials, fresh egg white started to denature when heating temperature was higher than 65 °C. When heating temperature was at 55 °C, no obvious aggregation was observed even after 4 h. As shown in Figure 5, by the whipping test, the non-heated egg white had significantly better foam expansion than the treated samples. For foam stability, except for the sample with holding

time of 30 min which showed a difference compared with the control, all others did not show a significant difference. For foam liquid stability, all heat-treated samples showed a poorer performance than the control. Based on the parameters of foam expansion and foam liquid stability, we concluded that at 55 °C, holding time of 10 min reduced foaming properties significantly and prolonged holding caused the similar damage as that of holding time of 10 min.

Effect of heating at higher temperature (62 to 64 °C) on foaming properties

At higher temperature with short holding time as seen in industrial HTST operation, increased protein aggregation was observed with prolonged holding time. As shown in Figure 5, foaming expansion had no significant change within 3 min as compared to the control. Starting from 4 min, foam expansion reduced sharply from almost 800% to below 200%, meanwhile apparent protein aggregation was observed. Foam stability showed a similar change over time and downward slope was seen after 3 min with a significant decrease seen in 5 min. Foam liquid stability slightly improved over time within the first 3 min though it was not significant, and it was quickly increased after 3 min. This experiment showed that when holding time is longer than 3 min at 62 to 64 °C, all parameters showed poorer values in comparison with the control.

Changes in egg functionality caused by heat treatment have been a major concern since pasteurization of liquid egg products became mandatory in 1970s in the United States. Foaming change was the 1st noticeable phenomenon among its many applications when egg white is heated (Stadelman and Cotterill 1995). Our study provides quantitative analysis of how heating affects foaming.

Effect of industrial processing on foaming properties of liquid egg white

Liquid egg white samples were taken from different processing steps of a modern egg breaking facility, Sparboe Foods Corp. (New Hampton, Iowa, U.S.A.). Samples were stored at 2 °C in a refrigerator and tested within 24 h. The results are shown in Figure 6. Sample A was taken from the

process point where egg white just went through the filter disk. Sample B was taken before the egg white went into pasteurizer where it went through 2 pumps, transfer pipe, heat exchanger, and balance tank. Sample C was at the step right after pasteurization at 57 °C for 3.5 min holding time. It should be mentioned that egg white taken from the plant was not as pure as the sample we separated in the laboratory, which was used as the control. So it is not surprising that all 3 samples showed poorer foaming properties than the control, as we have proved that even very low level of egg yolk contamination caused a significant reduction on foaming properties of egg white protein. Also for these 2 parameters (F_c , V_i), sample B did not show a difference compared to sample A. This indicates that shearing during liquid transfer did not significantly impact foaming properties. However, after pasteurization both F_c and V_i increased significantly as compared sample A and sample B. This suggests that either the shearing or the heating from the pasteurization has partially unfolded the proteins and improved foaming properties.

Results by the whipping method showed a similar trend in foaming properties as measured by nitrogen sparging method. However, the whipping method gave a significant difference between sample A and B, and such a difference was not shown by the sparging method. This indicates again that whipping is a better method in evaluating foaming.

Conclusions

The results of this study confirm that the whipping method is consistent with the nitrogen sparging method in general, and the results also indicate that shearing and heating as encountered in the egg breaking and pasteurization facility do not cause significant foaming reduction. Any yolk contamination of the egg white has the most significant impact on foaming properties.

Acknowledgments

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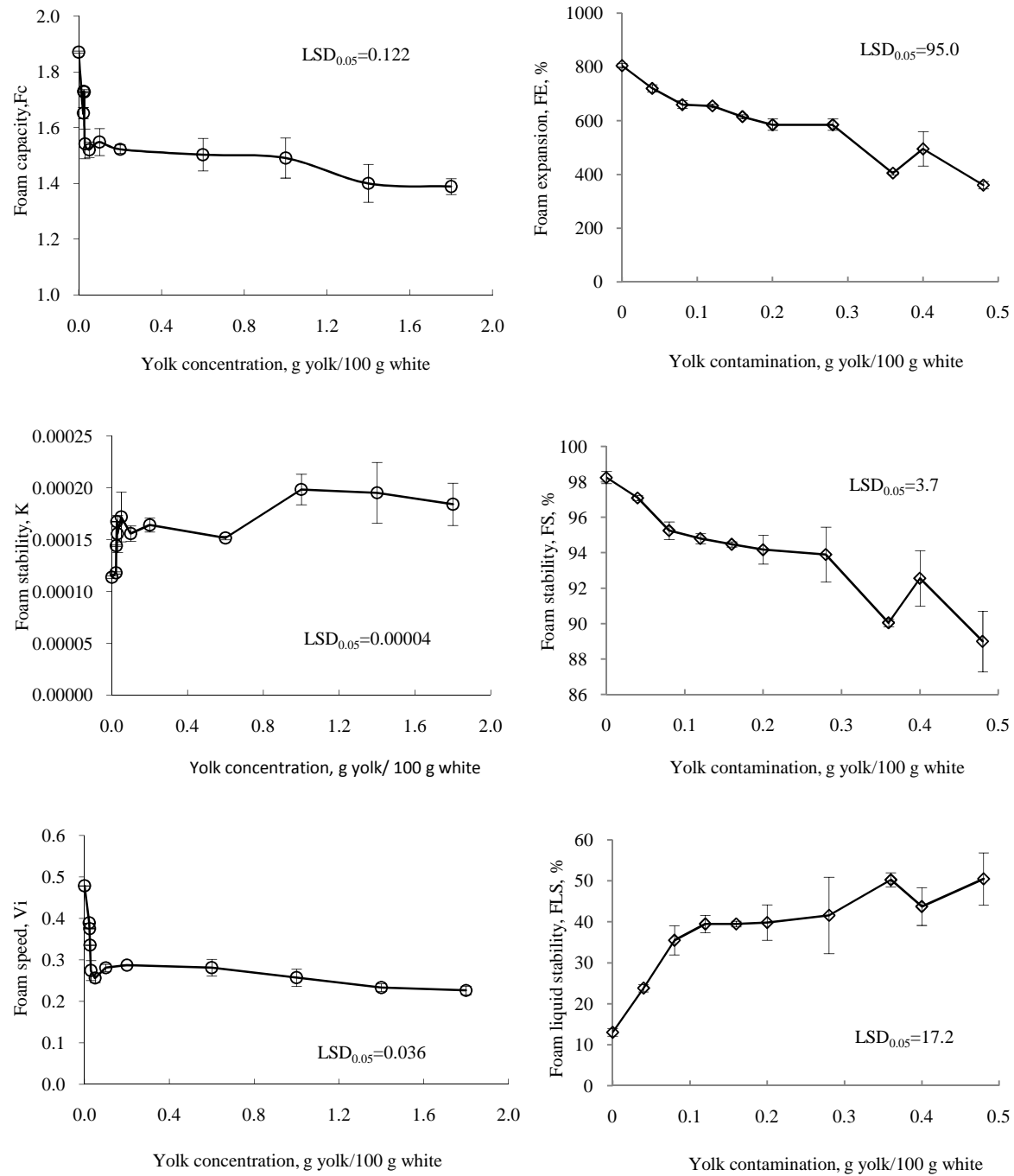


Figure 1. Foaming properties of yolk-contaminated egg white as measured by nitrogen sparging method (left column) and whipping method (right column). The vertical bar on each spot represents the standard deviation of duplicate measurements.

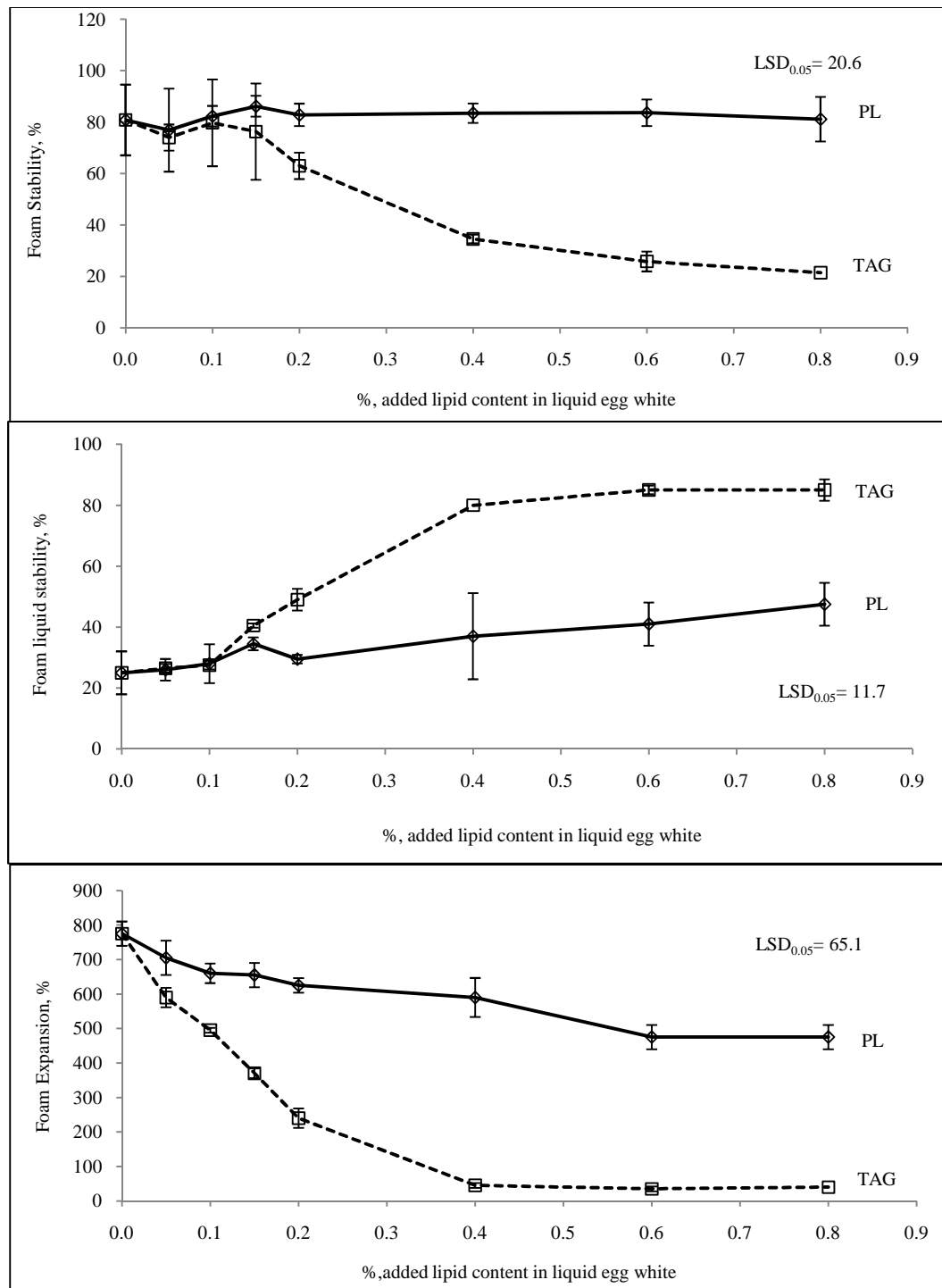


Figure 2. Foaming properties of egg white as affected by adding different lipid fraction of yolk lipids, measured by the whipping method (PL: phospholipids, TAG: neutral lipid).

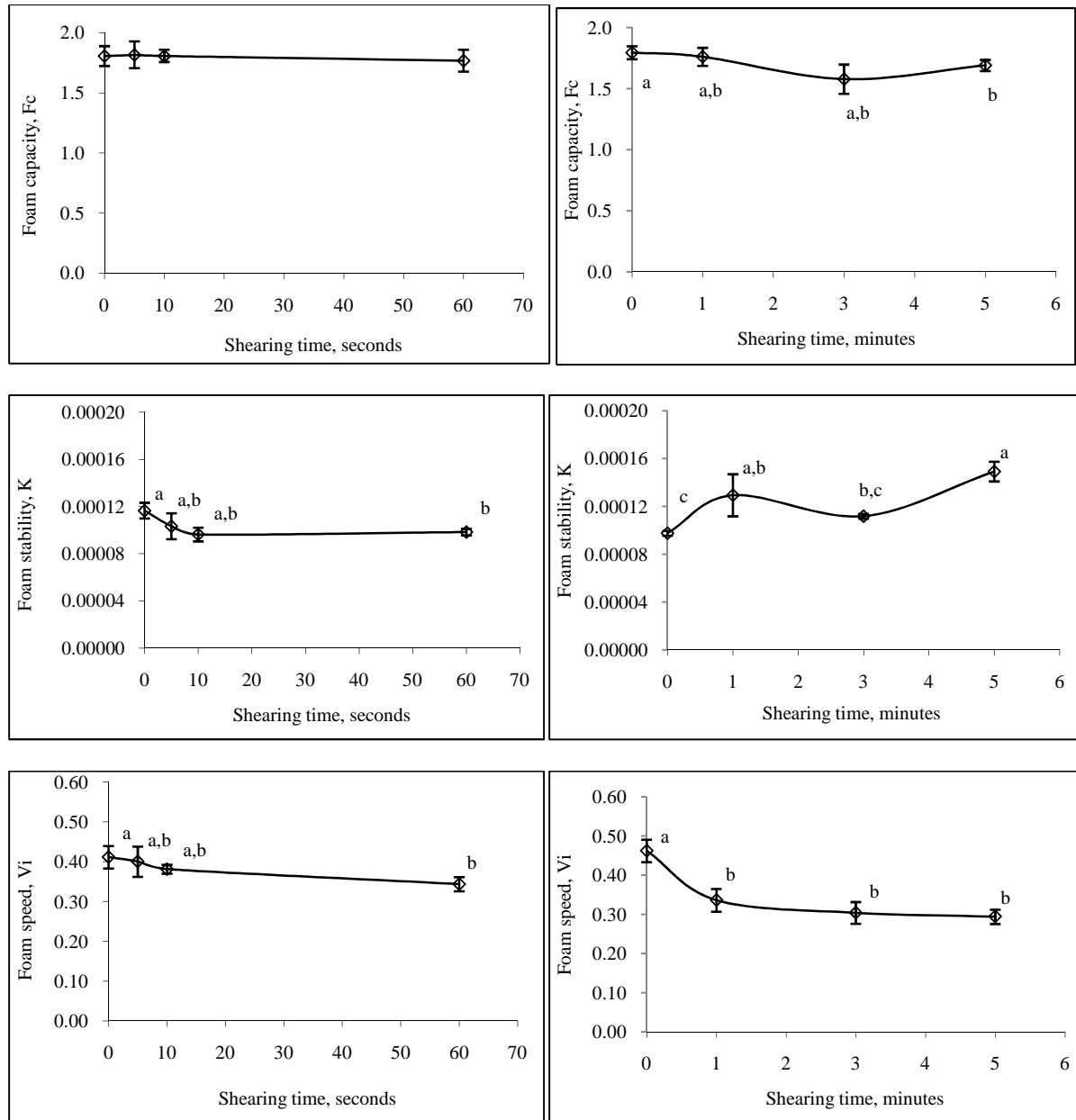


Figure 3. Changes in foaming capacity (F_c), foaming stability (k), and foaming speed (V_i) as affected by shearing at 14,000 rpm (left column: short time shearing, right column: prolonged shearing), measured by sparging method. Different letters above each treatment represent significant difference at 95% confidence interval.

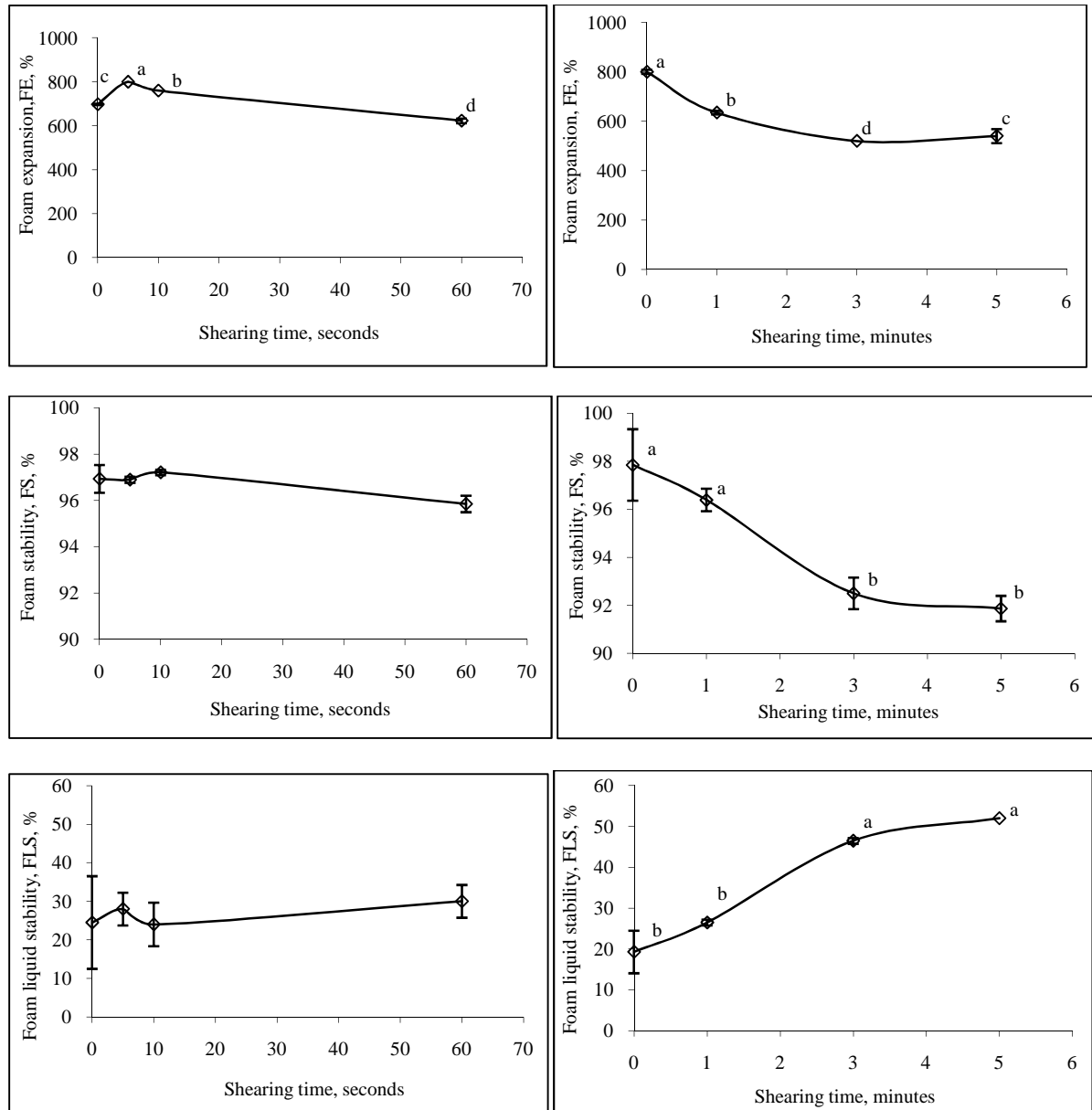


Figure 4. Foam expansion (FE), foam stability (FS), and foam liquid stability (FLS) as affected by shearing at 14,000 rpm (left column: short time shearing, right column: prolonged shearing), measured by whipping method. Different letters above each treatment represent significant difference at 95% confidence interval.

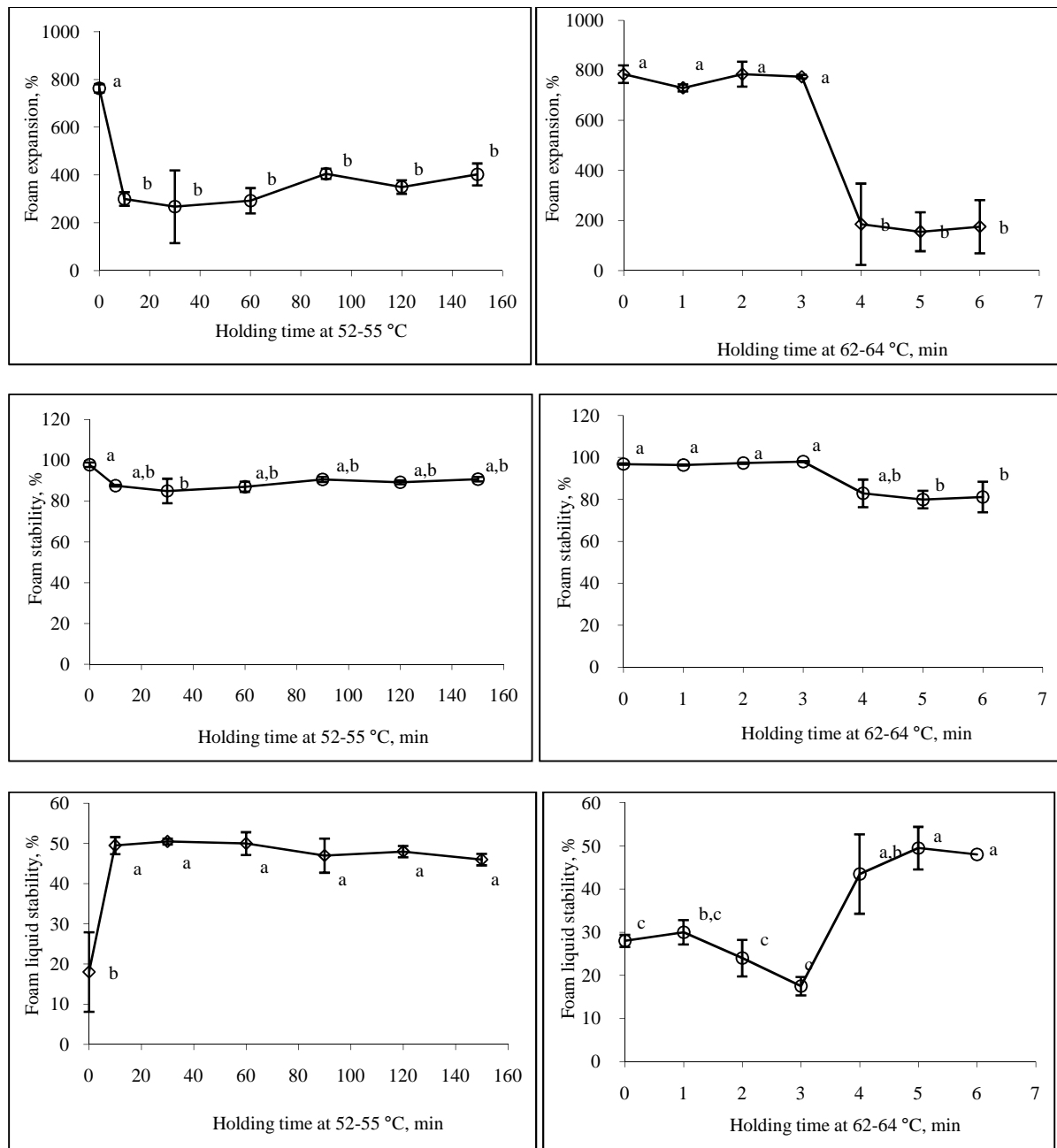


Figure 5. Egg white foaming as affected by thermal treatments (left column: 52-55 °C, right column: 62-64 °C) as measured by whipping method. Different letters above each treatment represent significant difference at 95% confidence interval.

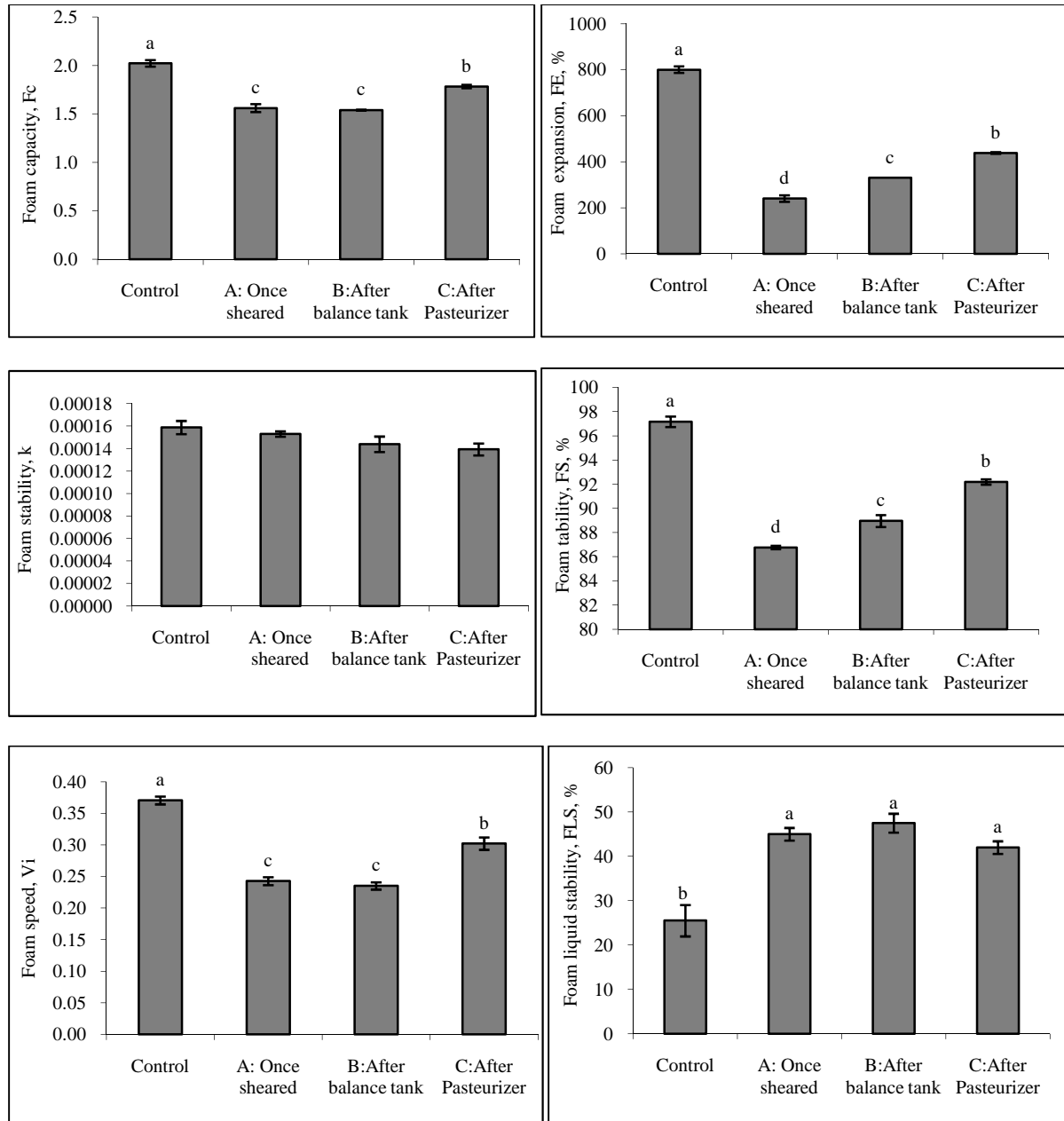


Figure 6. Foaming properties of liquid egg white as affected by plant processing (figures on the left side was measured by sparging method and figures on the right side was measured by whipping method). Different letters above each treatment represent significant difference at 95% confidence interval.

CHAPTER 5. IMPROVING FOAMING OF YOLK-CONTAMINATED EGG WHITE BY ADDITION OF BASIC PROTEIN

A paper adapted from the publication in the *Journal of Food Science*¹

Guang Wang² and Tong Wang^{2,3}

Abstract

Yolk contamination of egg white is a common problem in the egg breaking industry. Foaming properties of egg white protein are affected by such contamination, but proteins of basic nature may restore the foaming properties of the yolk-contaminated egg white protein. The purpose of this study was to chemically modify a soy protein, that is, to esterify the acidic groups on the protein and to study the potential of such modified protein in improving foaming. We showed that the modification changed the isoelectric point of soy protein isolate (SPI) from 4.5 to about 10. Sonication was proven to be a very effective means to re-disperse the methanol-denatured soy protein during reaction, as shown by the improved solubility profile. Such modified basic protein, that is, the sonicated-modified SPI (SMSPI), when added to the yolk-contaminated (at 0.4% level, as-is basis) egg white, gave significantly improved foaming properties. The slight change in pH due to the addition of SMSPI was not the reason for improved foaming performance; instead, the modified protein itself was the main reason for such improvement. Addition of SMSPI increased the foaming performance of both pure egg white and yolk-contaminated egg white. SMSPI consistently performed better than the unmodified SPI for improving foaming. Addition of SMSPI (16%, based on dry egg white, and 1.6% based on liquid egg white) fully restored foam expansion and foam liquid stability of

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² Department of Food Science and Human Nutrition

³ Author for correspondence

0.4% yolk-contaminated egg white, and it even out-performed the foaming of pure white protein. Therefore, a feasible solution to restore the foaming properties of yolk-contaminated egg white has been identified. It is expected that such modified SPI can be used as an additive or ingredient in foaming formulation, especially when the egg white protein is suspected of lipid contamination.

Keywords: Basic protein, foaming properties, foaming restoration, sonication, yolk-contaminated egg

Introduction

Egg white albumen has been widely used in food application because of its excellent functional properties, such as foaming, emulsification, and gelation. Yolk contamination of egg white reduces the albumen's functional properties, especially the foaming performance, and this contamination may not be completely preventable even with the modern egg breaking process, as shown in our previous study (Wang and Wang 2009). The extent of yolk contamination may vary from 0.01% to 0.2% depending on specific operational conditions and raw material quality (Cotterill and Funk 1963; Stadelman and Cotterill 1995). Even with 0.01% contamination, egg white showed reduced foam expansion (Wang and Wang 2009).

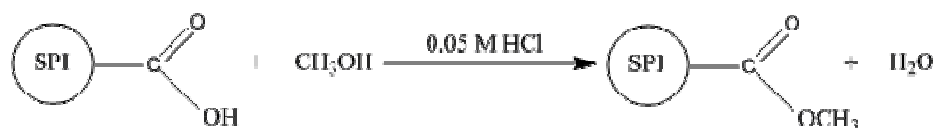
Protein forms and stabilizes foams by rapid absorbing and unfolding at the interface of air and liquid, and forming a cohesive film (Damodaran 1990). The visco-elastic film at the interface is attributed to protein–protein interaction, such as electrostatic and hydrophobic interactions (Poole and others 1984; Liang and Kristinsson 2005). Structural modification of proteins might happen under heating or high shearing condition (Kitabatake and Doi 1987; Van der Plancken and others 2005), thus affecting protein unfolding and interactions, and, ultimately, foaming. Egg white albumen is a complex mixture of proteins and each protein component possesses a different isoelectric point (pI), such as ovalbumin (54% of total protein) with pI of 4.5, ovotransferrin (12%) with pI of 6.1,

ovomucoid (11%) with pI of 4.1, globulins (8%) with pI of approximately 4.8 to 5.5, and lysozyme (3.4%) with pI of 10.7 (Li-Chan and Nakai 1989). Because of its diversity in protein composition, egg white tends to carry certain charges in a wide pH range, although the majority of the individual proteins carry negative charge at neutral pH.

The application of basic proteins to an acidic protein to improve foaming properties has been studied by several workers (Poole and others 1984, 1986; Phillip and others 1989; Poole 1989). Adding clupeine (pI of 12) and lysozyme with low concentration (0.01% to 0.10%, w/v) to several acidic proteins greatly improved their foaming properties, with clupeine being more effective (Poole and others 1984) than lysozyme. Lysozyme (0.1%) failed to promote foaming when added to an egg albumen and ovalbumin at a concentration of 0.5%. In the presence of certain lipids (5% corn oil or butterfat oil), clupeine and lysozyme also restored foaming properties of whey proteins (Poole and others 1986). The mechanism of basic proteins improving foaming is that they neutralize the negative charges of the egg albumen, leading to a stronger protein–protein hydrophobic interaction, or that the oppositely charged proteins have a stronger electrostatic interaction, thus increasing protein film strength.

Commercial utilization of the effective and naturally occurring clupeine and lysozyme is limited because of their rare availability and high cost (Malamud & Drysdale 1978; Poole 1989). Chemical modification to make basic protein or polysaccharide becomes attractive because the reaction is relatively easy (Fraenkel-Conrat and Olcott 1945; Means and Feeney 1971). Poole (1989) tested lipid tolerance of whey protein systems by adding basic whey protein and chitosan, both of which were prepared by chemical modifications, and similar results were reported as seen in clupeine and lysozyme studies. However, a lecithin-containing protein system still showed resistance to chitosan addition for foaming restoration. Few studies have been conducted to investigate how yolk-contaminated egg white system responds to the use of modified basic proteins.

Soy protein (i.e., SPI, soy protein isolate) is a good candidate for protein modification because of its availability and price. Therefore, soy protein was used as a model in this study. The same reaction approach can be applied to other proteins, especially the egg white protein, and with various types of alcohols. The modification reaction, that is, esterification, is simplified as shown below (Means and Feeney 1971):



The objectives of our study were to make a basic protein from a SPI and to evaluate the foaming properties of yolk-contaminated egg white with the addition of this chemically modified soy protein.

Materials and Methods

Preparation of basic soy protein isolate by esterification

SPI was provided by Archer Daniels Midland (Decatur, Ill., U.S.A.). It had a moisture content of 4.5% and was dried for 72 h using a vacuum oven at 40 °C. The residual moisture was measured as less than 0.2%. Dried SPI of 10 g was dispersed in 500 mL methanol (moisture content less than 0.05%). Concentrated hydrochloric acid of 2 mL was measured into the flask, which gave a final concentration of 0.05 M HCl in methanol. The esterification reaction was carried out under ambient temperature (25 °C) with magnetic stirring for 48 h and the reaction was stopped by adding 1 mL deionized water. The product was cooled to 4 °C and the solid was collected by filtration. The solid was pre-dried in a fume hood overnight, and the residual solvent and moisture were removed overnight using a vacuum oven (National Appliance Company, OR) at ambient temperature. The resulting product was ground using a mortar and pestle and stored in a desiccators. This modified SPI was designated as MSPI.

Protein sonication

Protein samples (SPI and MSPI) were suspended in deionized water with a concentration between 20 and 25 mg/mL. Sonication was carried out for 15 min at setting 9 (power output of 540 W at 20 KHz) with Pulsar on 60 s every 2 min using a Misonix XL Sonicator (Farmingdale, N.Y., U.S.A.). Samples were surrounded by an ice–water bath to avoid overheating. This sonicated MSPI was designated as SMSPI, and sonicated SPI was designated as SSPI. The pH of the sonicated protein dispersions was adjusted to 11 by adding 1 N sodium hydroxide before it was added into liquid egg white to obtain maximal solubility.

Protein solubility determination

A series of 1% (w/v) protein aqueous dispersion with pH from 2 to 12 was prepared. The pH of these dispersions was adjusted using hydrochloric acid (0.1 N) or sodium hydroxide (0.1 N) solutions. Protein dispersion was then centrifuged at 4,000 *g* for 10 min using a IEC Centra CL3 centrifuge (Thermo Electron Corp., Waltham, Mass., U.S.A.). Solubility was determined by quantification of the protein in the supernatant using the Biuret colorimetric protein assay with absorption wavelength at 540 nm (Gornall and others 1949). The bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was used to establish the standard curve for protein quantification.

Foaming properties of egg white as measured by whipping method

Fresh hen eggs purchased from a local grocery store were cracked and the egg white was manually separated from the yolk without any contamination. The solid content of the egg white was measured as 10% (weight base), and it had a pH of 8.4 to 8.8. Liquid egg white suspension (5% albumen solid) was prepared as a control. Contaminated egg white was prepared by adding 0.4% fresh egg yolk (w/w, in fresh egg white). The foaming properties were measured by a whipping test as described by Poole and others (1984) with minor modification. Briefly, whipping was performed at a

speed setting of 10 (400 rpm) for 90 s with a KitchenAid brand mixer attached with a wire whip. All treatments were done using 100 mL dispersions of 5% base protein concentration. The initial foam volume was measured by gently filling and tapping the foam into a 1 L graduated cylinder. The final foam volume and the liquid volume drained from the foam after 30 min were also measured for calculation of foaming properties. To measure the volume after 30 min standing, drained liquid was first poured out and measured, and the foam was measured with gentle tapping for even foam packing in the 1 L graduated cylinder. A total of 3 foaming parameters were calculated from the data collected:

Foam expansion (FE), % = $100 \times (\text{total volume of foam and liquid} - \text{initial volume of liquid}) / \text{initial volume of liquid}$;

Foam stability (FS), % = $100 \times (\text{foam volume after 30 min} / \text{initial foam volume})$; and

Foam liquid stability (FLS), % = $100 \times (\text{volume of liquid drained from foam after 30 min} / \text{initial volume of liquid})$.

Effect of pH change on foaming properties of yolk-contaminated egg white

Liquid egg white containing 0.4% (w/w) yolk was divided into 2 groups: in the 1st group, various amounts (2%, 8%, and 16% based on solid egg white albumen) of SMSPI were added to the yolk-contaminated egg white in the form of 20 mg SMSPI/mL stock solution as it was done in previously mentioned experiment. Then, deionized water was added to a total weight of 100 g. The pH of these treatments was measured as 9.1, 9.4 and 9.7, respectively; Treatments in the 2nd group did not contain any SMSPI besides egg white, but pH was adjusted to the same level as that in SMSPI group using 0.1N sodium hydroxide solution. Another 2 samples, egg white and egg white with 0.4% of yolk were prepared as controls with a pH of 8.8 for both. Foaming properties of these samples were then measured.

Effect of commercial SPI, sonicated SPI, modified SPI, and SMSPI on foaming properties of pure egg white and contaminated egg white

The 4 SPI stock solutions with protein concentration of 20 mg/mL and pH of 11.6 were prepared from SPI, sonicated SPI (SSPI), modified SPI (MSPI), and SMSPI. Forty mL of each stock solution were added into 50 g of fresh egg white, which is equivalent to 16% soy protein relative to egg white albumen. Yolk-contaminated egg white (0.4%) was used as the 2nd group with 2%, 8%, and 16% addition of previously mentioned 4 SPI samples. Final volume of each sample was adjusted to 100 mL with deionized water. Foaming properties were measured by the whipping method. The pH of White control and Yolk control were 8.8 and pH of the other treatments were 9.1, 9.4, and 9.7 for 2%, 8%, and 16% SPI treatments, respectively, as shown in Figure 3.

Statistical analysis

The solubility curve of protein was done once but with duplicate measurements. All foaming treatments, except for the effect of pH experiment, were repeated twice with randomized complete block design (RCBD) (Cochran and Cox 1957). SAS 9.1 with analysis of variance (ANOVA) and general linear model (GLM) was used for data analysis, and mean comparisons were made at a confidence level of 95%.

Results and Discussion

Solubility and pI of the modified SPI and effect of sonication on protein solubility

Low protein concentration and a water-free environment are critical factors leading to maximum esterification reaction. As seen in Figure 1, reaction product prepared from 1% SPI gave a pI of about 11, whereas that from 2% SPI dispersion had a pI of about 9.5. This indicates that at high concentration, protein's active reaction sites may not be fully accessible to methanol. Poole (1989) mentioned that up to 5% whey protein was used when a mixture of propylene glycol and

isopropanol was used as reaction medium. Different solvents may have different optimal substrate concentrations. Poole's solvent mixture is less toxic than methanol, but the reaction time was much longer (4 to 6 d). Other solvents, including ethanol, will be further explored once this approach in this study is proven successful. The presence of trace amount of methanol in foods is a concern but in some cases it is natural and acceptable as regulated by FDA (CFR 21 – 173.250).

In the functionality study, all the modified SPI samples were made from 2% SPI dispersion preparation because of its high batch capacity and solvent use efficiency. Under such esterification reaction conditions, most proteins might have undergone considerable change in 3-D conformation because of the organic solvent denaturation of the water dispersible proteins; therefore, a greatly reduced solubility of the resulting protein is expected. As shown in Figure 1, the native SPI had pI of about 4.5, and pI increased to about 9.5 after esterification reaction, and its solubility was decreased compared to the unmodified SPI. Preliminary experiments revealed that directly adding such chemically treated SPI to yolk-contaminated egg white even further impaired foaming properties of the egg white. This suggests that the low solubility of such protein may limit its successful application in enhancing foaming. Sonication is known to be a powerful tool to break the intermolecular hydrophobic association of the denatured protein and therefore, to re-disperse protein. In comparison to hydrogen bond (10 to 40 kJ/mol) and electrostatic interaction (25 to 80 kJ/mol), the energy needed to break hydrophobic interaction (5 to 10 kJ/mol) is low (Eissa and Khan 2006). It is likely that the sonication treatment applied to the aqueous protein dispersion modified hydrophobic interactions of the denatured protein (Furukawa and Ohta 1983), in that large protein aggregates were broken into to smaller ones, thus protein became more dispersed. As shown in Figure 1, sonication caused little change in pI value. However, solubility of modified SPI after sonication (SMSPI) increased from 30% to about 85% within the acidic range (pH 2-7), and it was even higher for the SMSPI prepared from 1% SPI in methanol.

Effect of adding SMSPI on foaming properties of yolk-contaminated egg white

Foam expansion (FE) was used to characterize how much foam in volume is generated from the initial liquid after whipping. As shown in Figure 2A, adding 4% (w/w, basic protein in egg white albumen, dry weight basis) of SMSPI to pure white albumen gave slightly higher FE (880%) than that of egg white control (780%). For the yolk-contaminated treatments, FE steadily increased as the ratio of SMSPI to egg white albumen increased. Yolk-contaminated albumen without SMSPI addition (labeled as 0) had a FE value of only 545% compared with 780% of white albumen control. FE was completely restored when 4% of SMSPI was added with FE of 715% compared with the yolk-contaminated control. And FE of the last treatment (16% SMSPI) was 950%, which was significantly higher than that of white albumen control. Figure 2B shows foam stability (FS). This parameter failed to differentiate treatments of yolk control (with yolk but without SMSPI) and white albumen control. The experimental variability was too large to show meaningful significant differences among treatments for this parameter. The reason why FS of 4% SMSPI in yolk-contaminated white was different from the others is not clear. Very similar trend, as seen in FE, was observed for FLS as shown Figure 2C. This parameter represents liquid drain from foam after 30 min of standing. Treatment with 16% SMSPI had much less drainage (5%) in comparison with the 34% drainage of yolk-contaminated sample, and with 14% drainage of white albumen control. The basic SMSPI was shown to be very effective in reducing liquid drain from the foam.

All data in Figure 2 show that the foaming restoration power of SMSPI for yolk-contaminated egg white is dose-dependent, and addition of 8% to 16% of SMSPI can fully restore foaming properties, which were reduced by yolk contamination (at 0.4% level). In a real life situation, yolk contamination level may be much lower than what we used in this model system; therefore, much less SMSPI may be needed for foaming restoration. We used this high level of yolk contamination,

because at this level, foaming is significantly reduced, which will allow us to clearly demonstrate the improvement we can make by the treatments we designed.

Poole (1989) suggested that the ratio of basic to acidic protein is important in foaming because insolubilization of the protein prior to whipping may happen if too much basic protein is present in the systems. Poole's studies showed that a ratio of 1:10 in the case of clupeine (pI 12) was appropriate, and 3:10 was required for lysozyme (pI of 10.7) and a modified beta-lactoglobulin (pI of 8.1 to 9.9). A chemically modified basic protein based on whey protein isolate (pI > 9.5) was also successfully used at 0.3% to enhance the foaming of a 10% corn oil contaminated serum albumin protein but it was combined with 10% of sucrose (Poole and others 1986). We have shown in this study that much lower amounts of basic soy protein than the natural proteins can be used to effectively restore foaming properties of the heavily yolk-contaminated (0.4%) liquid egg white.

We have demonstrated that the SMSPI is an effective material to enhance foaming. However, several factors that are related to the preparation of basic SPI need to be examined to show how they may have contributed to the foaming properties of the egg albumen. pH values of these treatments were different because varied amounts of SMSPI were added. Also, it is important to know how the starting materials, native SPI, and sonicated SPI, affect egg white foaming if they were used without chemical modification.

Effect of pH change on foaming properties of yolk-contaminated egg white

As shown in Figure 3A, for group SMSPI, FE improvement is again positively related to dose increase and this is consistent with our previous results. On the contrary, the 3 treatments in the “water” group did not change as pH changed, and they had similar value to Yolk control. This result indicates that it is the addition of SMSPI, not the pH that contributes to the enhancement of FE. FS as shown in Figure 3B and FLS as seen in Figure 3C show the same trend as that of Figure 3A. Therefore, pH change alone did not improve FS and FLS of yolk-contaminated egg white, rather, SMSPI did,

and it had much higher FLS as the dose increased to 16%. All these observations clearly indicate that the improvement in foaming properties by SMSPI is truly a protein effect. These results are also consistent with what Chang and Chen (2000) found for liquid whole egg. After egg white experience unfolding at high (approximately 11 to 12) pH, consequently refolded egg white (pH 7.5) had a good foaming performance (Liang and Kristinsson 2005). However, the effect of refolding was not examined in this study.

It is worth mentioning that this experiment was done with one replicate. The trend of pH effect was studied, but not just for a single point or treatment comparison. Treatment with one replicate in such trend or relationship studies is generally considered acceptable, just as in protein solubility curve determination. Another experiment was done later to partially repeat the pH effect and similar results, as presented in Figure 3, were obtained. Since all proteins were from different batches, these data are not presented in this manuscript.

SMSPI was proven effective in restoring foaming properties of yolk-contaminated egg white, but it was unknown whether SMSPI is more effective than commercial SPI and sonicated SPI. If SPI is equally effective, the preparation of such protein might be much easier, for example, directly using SPI or just sonicating SPI. Two additional experiments were conducted to answer this question.

Effect of commercial SPI, sonicated SPI, modified SPI, and SMSPI on foaming properties of pure egg white

As shown in Table 1, at addition level of 16% (dry weight basis), these 3 treatments, SSPI, MSPI, and SMSPI had significantly higher FE than the White control. Sonication seemed to play an important role when SPI is compared with SSPI, and MSPI is compared with SMSPI. Sonication is believed to induce physical disruption and/or chemical transformations of the protein (El'Piner 1964). However, the effect of sonication on protein solubility and functional properties may vary depending on sonication treatment conditions, such as frequency used, treatment time, and protein concentration.

Both positive and negative results of sonication were reported in terms of solubility and functional properties of the resulting proteins (Wang 1981; Jambrak and others 2008). It is also interesting to note that treatment SSPI had similar performance to MSPI. In terms of FS, all samples showed no significant difference when compared with the White control, with only exception of SMSPI, which had lower stability. For FLS, the amount of drainage after 30 min of standing, all treatments had similar trends as found for FE. SPI had no difference from the White control, whereas all other treatments showed much better performance. There was no liquid drainage at all for both MSPI and SMSPI. With these results, we conclude that SPI itself did not enhance the foaming properties, whereas, modified SPI (MSPI at 16%) increased FE and FLS of pure egg white by 33% and 100%, respectively. SPI after sonication (SSPI) may have also interacted with pure white protein, thus improved FE and FLS by 30% and 76%, respectively. Further experiment was designed to demonstrate how foaming of yolk-contaminated egg white would respond to the addition of these treated proteins.

Effect of commercial SPI, sonicated SPI, modified SPI, and SMSPI on foaming properties of yolk-contaminated egg white

Pure white and yolk-contaminated white were prepared as controls. For all four SPI treatments, adding up to 2% soy protein started to show improved FE in comparison with Yolk control as seen in Figure 4A. Adding 8% soy proteins restored FE to the level of the White control. All 3 protein treatments, SSPI, MSPI, and SMSPI performed much better than SPI and White control at the high protein level of 16%. For FS, it was found that this parameter was not as sensitive as FE and FLS because treatments of White and Yolk controls did not even show a difference. Adding 2% of all treated proteins improved FLS as compared with Yolk control except for MSPI as shown in Figure 4C. In both 8% and 16% group, FLS was enhanced with increasing order of SPI, SSPI, MSPI, and SMSPI within each concentration level. All samples in both groups had significant

improvement in FLS as compared with Yolk, even with White control. This result is consistent with what was shown in FE. Treatment with SMSPI at 16% concentration showed both highest FE (1075%) and best FLS (0%). When this result is compared with what is presented in Table 1, where pure egg white was used instead, it is clearly demonstrated that for treatment with 16% SMSPI, almost 88% of FE and 100% of FLS caused by adding 0.4% yolk were restored.

The possibility of adding protein thus increasing viscosity may be a reason for reduced foam liquid drainage was also tested as a mechanism of foam stabilization. With the addition of 16% SPI and SMSPI to the 0.4% yolk-contaminated egg white, these dispersions had the same viscosity as that of the control (without any additional protein addition, data not shown). Therefore, viscosity is not a factor in foam stabilization in this study.

It is worth discussing whether the foaming improvement is due to the increased SMSPI protein concentration in the system, or it is truly due to the protein modification. When comparing the effects of SPI and SMSPI as shown in Table 1 and Figure 4 at various levels in the presence and absence of yolk contamination, it seems that 16% SPI addition did not improve foaming nearly as much as the 16% SMSPI, suggesting that SMSPI's effect on foaming may be mainly because of the structural modification. However, the fact that the sonicated SPI also gave foaming improvement may indicate that a properly dispersed protein also contributes to foaming, possibly due to concentration or other protein-protein interaction. More in-depth study is needed to quantitatively describe each of the contributing factors.

Conclusions

Chemical modification of soy protein was successful in making basic protein. Sonication was proven to be an effective method to re-disperse the methanol-denatured soy protein, and the resulting protein showed great ability in restoring foaming properties of yolk-contaminated egg white. This restoration ability directly correlated with the concentration of the basic protein used.

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Table 1. Foaming properties of pure egg white with addition of various processed soy protein isolates

Treatment*	Foam	Foam	Foam liquid
White only	810 ± 56.6	85.5 ± 9.2 ^a	17.0 ± 2.8 ^b
SPI	845 ± 7.1 ^c	91.0 ± 3.7 ^a	14.5 ± 0.7 ^b
SSPI	1100 ± 0.0 ^k	89.6 ± 2.9 ^a	3.5 ± 0.7 ^c
MSPI	1125 ± 35.4 ^k	86.2 ± 7.7 ^a	0.0 ± 0.0 ^c
SMSPI	1225 ± 35.4 ^k	68.3 ± 3.0 ^b	0.0 ± 0.0 ^c
Yolk	545 ± 35.4 ^d	77.3 ± 6.7 ^{ab}	31.5 ± 6.4 ^a
LSD	83.6	14.8	7.0

*All treatments had no yolk addition except yolk control. Addition of all SPI treatments was 16%

based on solid egg white albumen. Different letters represent significant difference at $P < 0.05$. White, egg white only, 5% solid egg white albumen; SPI, soy protein isolate; SSPI, sonicated SPI; MSPI, modified SPI without sonication treatment; SMSPI, modified SPI with sonication treatment; Yolk, egg white with yolk contamination of 0.4% (w/w).

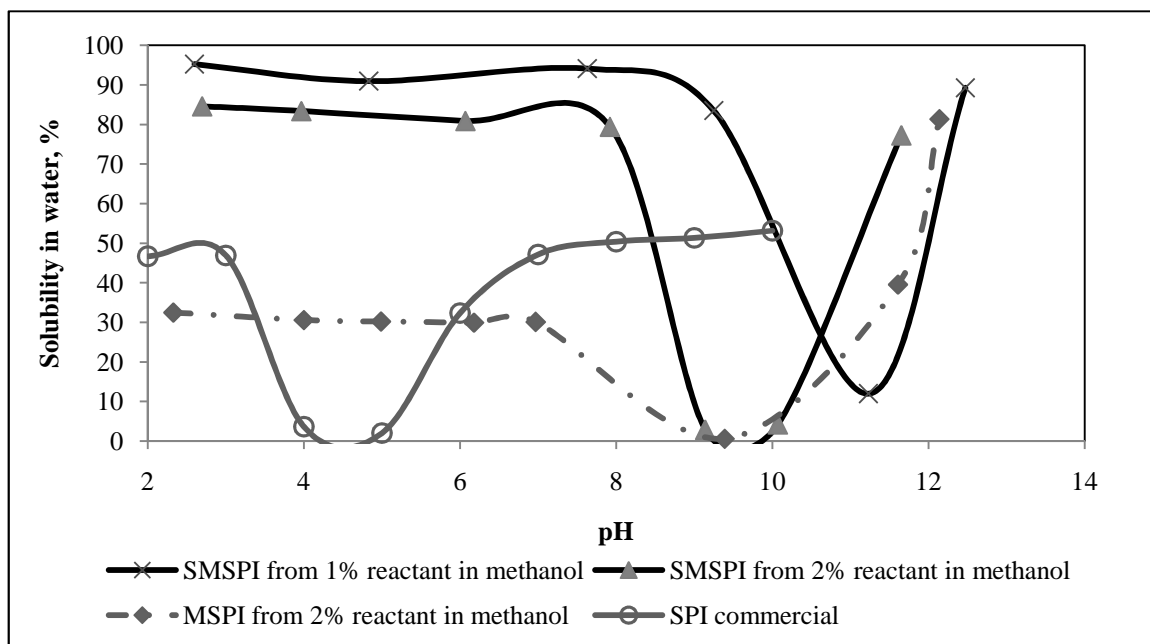


Figure 1. Solubility of soy protein isolates with chemical modification and sonication treatment. SMSPI, modified SPI with sonication treatment; MSPI, modified SPI without sonication treatment.

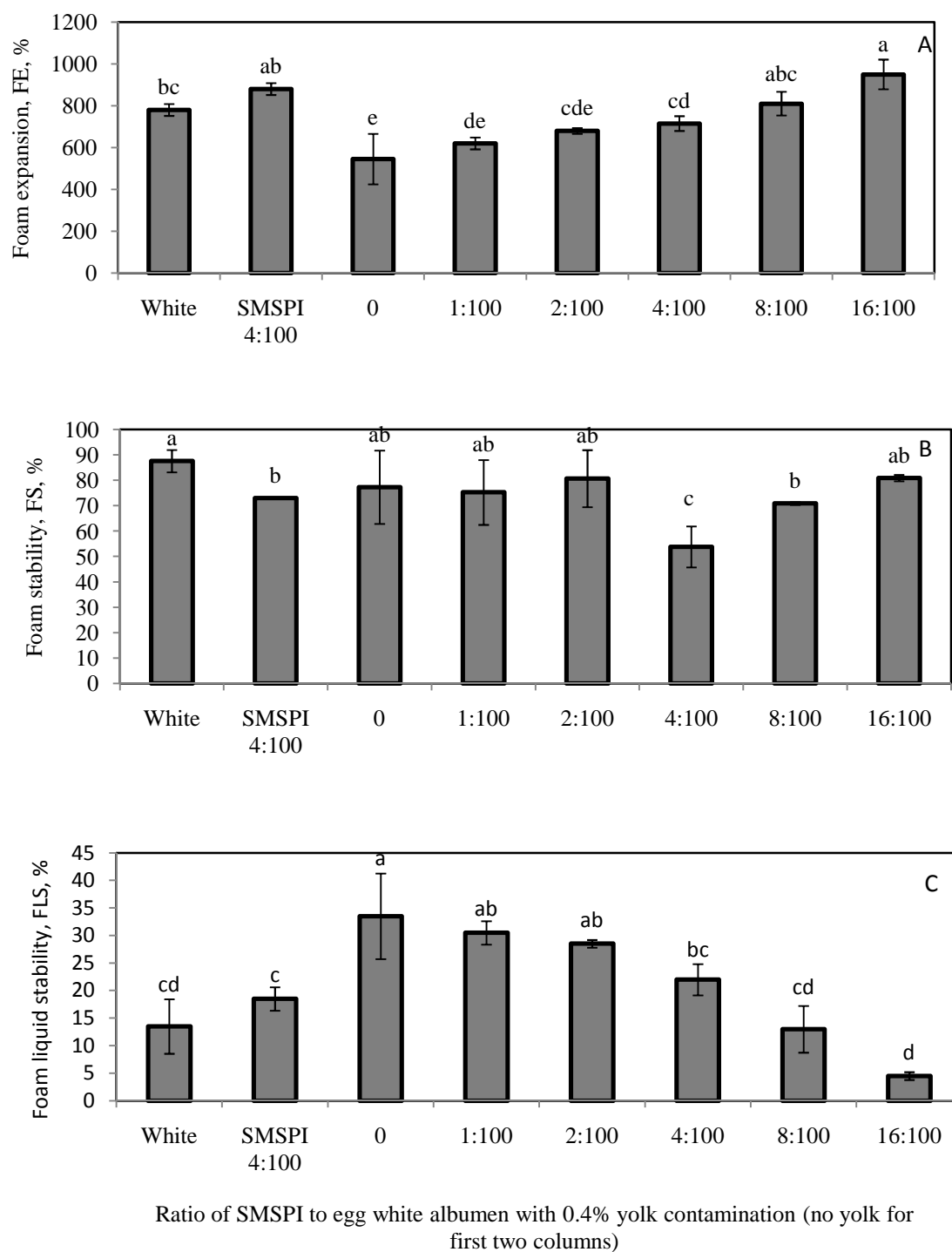


Figure 2. Foaming performance of yolk-contaminated egg white solution as affected by adding various amount of basic soy protein isolate. Different letters on the bars represent significant difference at $P < 0.05$.

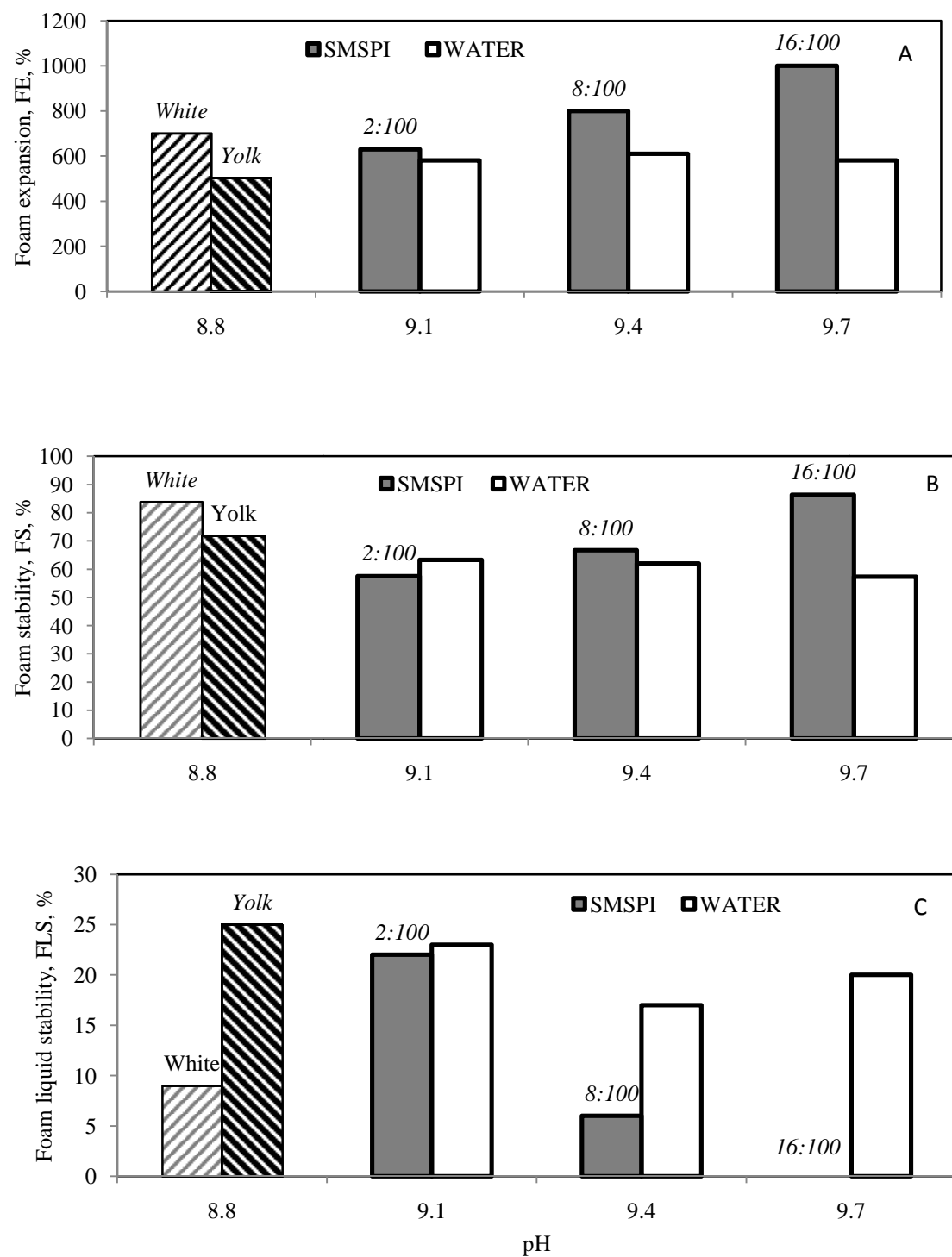


Figure 3. Changes in foaming properties of yolk-contaminated egg white as affected by SMSPI addition as compared with its pH controls.

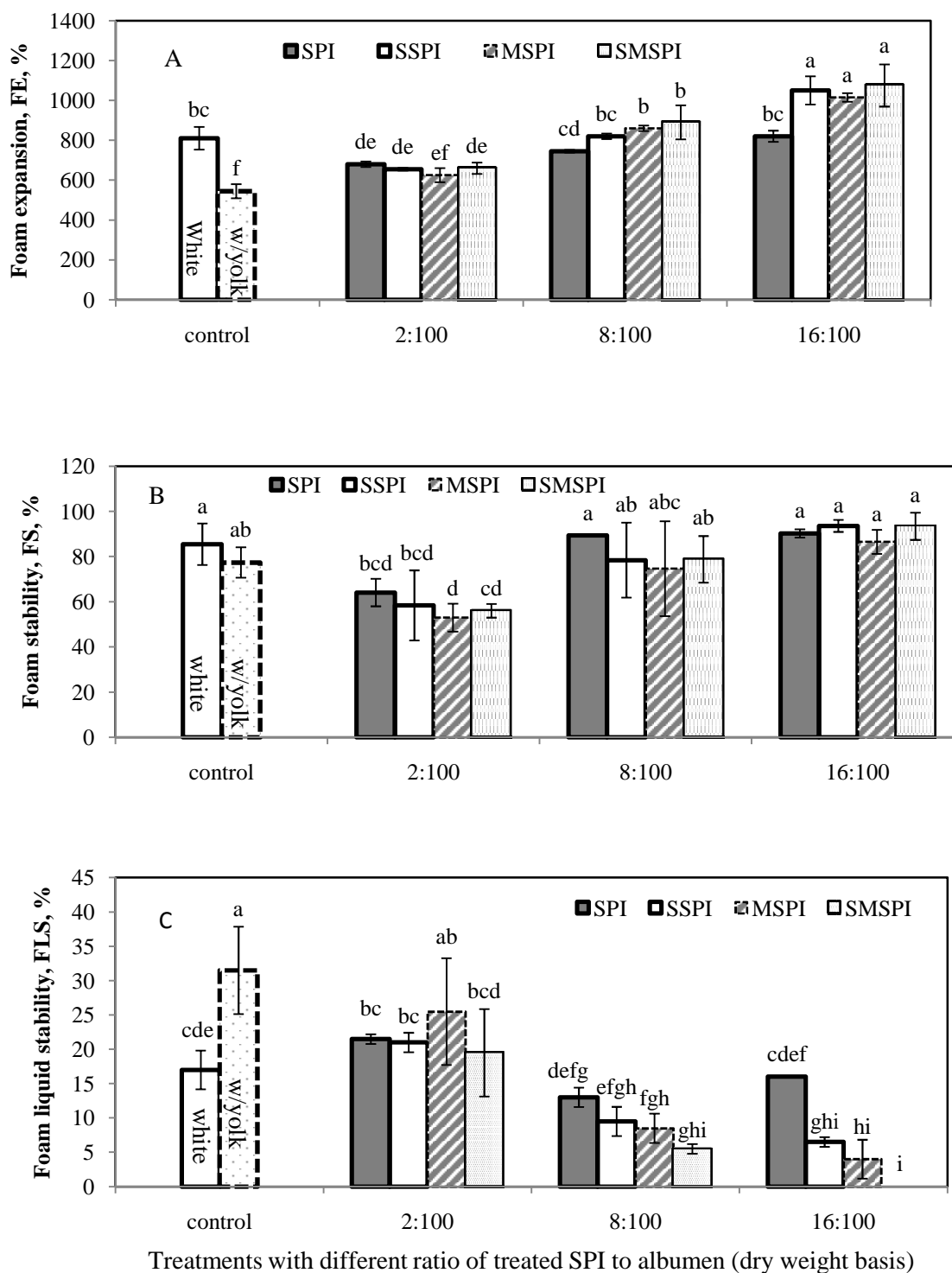


Figure 4. Foaming properties of yolk-contaminated white as affected by type and concentration of various soy proteins. For abbreviations of SPI treatments, refer to Table 1 footnote.

CHAPTER 6. GENERAL CONCLUSIONS

The essential theme of this dissertation focuses on better utilization of egg products and exploration and resolution of problems associated with functionalities of egg lecithin and egg protein in food. Egg provides both high quality protein and lipids, especially lecithin. Egg lecithin is the second major source for commercial lecithin, and its application covers foods, cosmetics, and pharmaceutical fields. One of the main functionalities of egg albumen protein in food preparation is its excellent foaming property as seen in various foamy food products, such as meringues and angel food cakes.

A major concern in utilizing egg lecithin as an emulsifier in food emulsions is lipid oxidation. Lipid oxidation causes off-flavor and losses in nutritional quality. In emulsion systems, multiple factors could affect oxidative stability of lecithin of both soy and egg sources. In our study, the role of transition metal ions, cupric and ferric ions, in oxidative stability of egg and soy lecithin was evaluated in emulsions. The effect of pH on lipid oxidation was also examined under two concentrations for each ion (50 μ M and 500 μ M). For the source of EL and SL used in the studies presented in Chapter 2, it was found that egg lecithin (EL) had a similar peroxide value (PV) development pattern as soy lecithin (SL) when treated with cupric ion under both acidic and neutral pH. Acidic pH of 3 accelerated oxidation of both EL and SL, especially under high concentration of cupric ion. When treated with ferric ion, EL oxidized much faster than SL. EL had higher secondary oxidation products (TBARS) than SL. Acidic pH accelerated TBARS development for both EL and SL, but EL had more significantly increased values. Cupric ion was more powerful than ferric ion in catalyzing oxidation of both EL and SL under both acidic and neutral pH conditions as measured by PV and TBARS. Overall, SL showed better oxidative stability than EL under our experimental conditions. This study also suggests that using multiple methods is necessary in properly evaluating

lipid oxidative stability, especially in the situation where lipid hydroperoxides tend to be easily decomposed.

As a minor component in egg lecithin, ethanolamine plasmalogen attracted our attention because of the increased interest in studying its antioxidant effect in an *in vivo* and *in vitro*. The reported antioxidant effect might have contributed to the better oxidative stability of EL than SL as we previously found in bulk oil. To better understand the role of ethanolamine plasmalogen in maintaining oxidative stability in *in vitro* system, an EthPm extracted from bovine brain (BBEP) was used in our oxidation study. It was found that in the purified soybean oil (PSO) system, the addition of 200 and 1000 ppm BBEP promoted lipid oxidation, whereas SL added at the same amount showed a similar oxidation trend to the PSO control. The more BBEP is added, the faster the lipid oxidation. Lipid oxidation of BBEP treatments was even faster in the presence of cupric ion than its non-cupric counterparts. In commercial soybean oil (CSO) with the presence of tocopherols, SL at 1000 ppm as an antioxidant acted synergistically with the natural tocopherols, but the addition of BBEP accelerated lipid oxidation as evidenced by the oxidative stability index (OSI). In the model liposome system, BBEP-added egg PL (EPL) liposome tended to have a fast breakdown of the lipid hydroperoxides, consequently promoted more secondary oxidation products. The oxidation of liposome lipid in the presence of copper was not affected by the presence of BBEP, which indicates that the hypothesis of ethanolamine plasmalogens chelating cupric ion as the antioxidant mechanism was not supported. The addition of cumene hydroperoxide to the EPL liposome promoted lipid oxidation as reflected by a very fast development of PV and TBARS. However, our study failed to differentiate the effect of BBEP and SL and their concentration on oxidation. It can be concluded that EthPm is not an antioxidant but rather a pro-oxidant in bulk lipid systems, whereas addition of EthPm has no significant effect on oxidation in liposome PL.

While egg lipid is of a concern due to its oxidative stability when used in emulsified food, yolk lipid contamination to egg white is another issue involved in egg protein foaming. Our study revealed that a concentration as low as 0.022% (as-is basis) of yolk contamination caused significant reductions in foaming capacity and foaming speed. Further test confirmed that it is the neutral lipid fraction of egg yolk causing the major detrimental effect on foaming, and the phospholipids fraction did not give significant foaming reduction at a concentration as high as 0.1%. High-speed and short-time shearing caused no apparent damage but longer shearing time significantly impaired foaming. Foaming was significantly reduced at a temperature of 55 °C for 10 min, whereas it did not change up to 3 min at a heating temperature of 62 to 64 °C. Industrial processing steps (pumping, pipe transfer, and storage) did not produce negative effects on foaming of the final products and the controlled pasteurization was actually beneficial for good foaming performance. Therefore, it is concluded that yolk contamination of the egg white was the major factor in reducing foaming properties of the white protein.

Foaming properties of egg white protein are affected by yolk lipid contamination, but proteins of basic nature may restore the foaming properties of the yolk-contaminated egg white protein. In this study, we also esterified the acidic groups on soy protein isolate (SPI) and studied the potential of such modified protein in improving foaming. It was shown that the modification changed the isoelectric point of soy protein isolate (SPI) from 4.5 to about 10. Sonication was proven to be a very effective means to re-disperse the methanol-denatured soy protein during the reaction condition, as shown by the improved solubility profile. The sonicated-modified SPI (SMSPI), when added to the yolk-contaminated (at 0.4% level, as-is basis) egg white, gave significantly improved foaming properties. Addition of SMSPI (16%, based on dry egg white, and 1.6% based on liquid egg white) increased the foaming performance of both pure egg white and yolk-contaminated egg white. SMSPI consistently performed better than the unmodified SPI for improving foaming. Addition of SMSPI

fully restored foam expansion and foam liquid stability of 0.4% yolk-contaminated egg white, and it even out-performed the foaming of pure white protein. Therefore, a feasible solution to restore the foaming properties of yolk-contaminated egg white has been identified.

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