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Egg yolk lecithin fractionation and characterization

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CHAPTER 1. GENERAL INTRODUCTION

Literature Review

The term lecithin, when used commercially, is generally described as a mixture of phospholipids (PLs), triacylglycerols, and glycolipids that is mainly derived from vegetable or animal sources. In other instance, lecithin is used scientifically as a synonym for phosphatidylcholine (PC), the predominant PL in most cell extracts. Soybean oilseeds are the major source of lecithin of vegetable origin, in which the major PL components are PC, 41-46%; phosphatidylethanolamine (PE), 31-34%; and phosphatidylinositol (PI), 19-21% (Cherry, 1989). The term lecithin can be traced back as Greek word for egg yolk, which was the first source of a lipid containing phosphorus, and it is actually the major animal source of lecithin (Horrocks, 1989). The main components of egg-yolk lecithin are PC, 80.5%; and PE, 11.7% (American Egg Board, 2002). Egg-yolk PLs represent approximately 10% of its wet weight, while soybean PLs represents only 0.40 to 0.45% of the seed weight (Chi and Lin, 2002). However, soybean lecithin is a by-product of oil processing, making it less expensive than egg-yolk lecithin. Nevertheless, neutral lipids and PLs from yolk are desirable because of the unique properties and valuable applications of these products (Sim, 1994). For example, the yolk oil is used in nutritional supplements, baby foods, or parenteral feeding because of the high ω-3 fatty acid content and the PLs are used in pharmaceutical and cosmetic industries. In addition, egg-yolk lecithin contains relatively more saturated fatty acids than soybean lecithin does; therefore, it may have better oxidative stability. It has also been shown that this more saturated egg-yolk lecithin has better plasma cholesterol lowering ability than the more unsaturated soybean lecithin (Jiang, *et al.*, 2001).

Egg-yolk Lecithin Composition and Structures

The main components of egg-yolk lecithin are PE and PC as already mentioned. Egg-yolk lecithin also contains lysophosphatidylcholine (LPC), phosphatidylserine (PS), sphingomyelin (SM), and minor quantities of neutral lipids. Figure 1 shows the structures of PLs in egg-yolk lecithin. In this thesis, the terms lecithin and PLs are used interchangeably. PLs consist of a glycerol and two fatty acids that are attached by ester bonds to the first and second carbon (sn-1 and sn-2 position). A phosphoric acid moiety is esterified with the hydroxyl group on the third carbon of the glycerol. Various polar head groups can be attached to the phosphate group, forming many PL classes. These molecules have hydrophilic heads and hydrophobic chains and therefore, are amphiphilic and can function as emulsifiers. PLs are widely used in many industries (Paltauf, 1990), primarily as surface active agents.

It has been suggested that the polar head groups give each PL class its distinctive functional property (Cherry, 1989). Other important factors determining the functional properties of PLs are the fatty acid chain length and degree of unsaturation of the fatty acids present in the molecule (Paltauf, 1990). PE of egg-yolk lecithin contained 18.5% palmitic acid, 23.9% stearic acid, 21.4% oleic acid, 13.7% linoleic acid, 13.8% arachidonic acid, and 3.0% docosahexaenoic acid (ω-3 fatty acid). PC of egg-yolk lecithin contained 34.0% palmitic acid, 1.7% palmitoleic acid, 11.0% stearic acid, 32.0% oleic acid, 18.0% linoleic acid, and 3.3% arachidonic acid (Avanti Polar Lipids, Inc, 2004). These values may change depending of the breed and feed of the hens.

Lecithin in Health and Nutrition

Lecithin is a good source of choline, a compound that plays an important role in cellular regulation and as a structural component of membranes and lipoproteins (Yen and Zeisel, 1998). Choline affects lipid transport and it is also the precursor of the neurotransmitter acetylcholine (Zeisel, 1998). A recently published research by Jiang, *et al.* (2001) reported that egg-yolk PC also lowered the lymphatic absorption of cholesterol in comparison to feeding the same amount of soybean PC to adult rats. The authors proposed that the hypocholesterolemic effect was the result of the inhibition of cholesterol absorption by the egg-yolk PC because of its higher degree of saturation of its fatty acids. The research on such hypocholesterolemic effect of egg-yolk lecithin is still very limited. More research needs to be done with animal models and humans to confirm this effect.

Functional Properties

Lecithin is a surfactant that has the ability to gather together in solution and form several thermodynamically stable structures such as micelles, bilayers, vesicles, and reverse micelles (McClements, 1998). Soybean lecithin is primarily used in the food industry, whereas egg-yolk lecithin is mainly used in pharmaceutical and cosmetic industries (Sim, 1994). In food industry, lecithin is used as emulsifier, stabilizer, wetting enhancer, and baking improver. In the pharmaceutical industry, PLs (in particular, egg-yolk lecithin) are used as aid in the delivery of drugs (Davis and Illum, 1993). This is because of the ability of PLs to form membranes (encapsulation), making PLs good constituents of drug vehicles especially in the formation of emulsions and liposomes (Davis and Illum, 1993).

The limited used of egg-yolk lecithin in food industry is because of the lack of a feasible processing technique and information on its functional properties. There is an urgent need to conduct such research and to increase the values of egg products.

Egg Yolk Fractionation

Egg-yolk lecithin is primarily extracted with solvents such as diethyl ether, hexane, chloroform, and ethanol. However, some of these solvents are considered undesirable because of environmental and health concerns (Sim, 1994; Nielsen, 2001). Earlier works in this area mainly focused on total lipid extraction and cholesterol removal and not on PL fractionation. Sequential extractions of total lipids with various solvents or multi-solvent system were investigated in the early 1980s (Larsen and Froning, 1981; Tokarska and Clandinin, 1985). PL fractionation techniques, such as ethanol extraction and low temperature crystallization to remove the solidified neutral oil (Sim, 1995), and ultrafiltration to isolate egg-yolk PLs (Miyata and Matsumoto, 2001) were also reported, but they had the disadvantage that they required specific equipment and temperature control. Hruschka *et al.* (2003) described a procedure of using low and high concentrations of aqueous ethanol in several steps to extract egg-yolk PLs, but they reported only 47% PL purity in the final product. At present, a rapid and economical method to produce yolk oil and/or lecithin directly from fresh yolk without using hazardous solvent is still needed.

The most common scheme of PL fractionation uses dried yolk and an initial deoiling step, and the PLs are then, extracted from the deoiled material with ethanol. Alternatively, dried yolk is extracted with ethanol first and then acetone is used to remove the oil from the ethanol extract. Dried yolk had been used as a starting material because of the general belief that lipid and protein in fresh yolk are intimately associated, and the lipids are not easily

extractable with non-polar solvent (Warren *et al.*, 1988). However, yolk drying is energy intensive and the heat-denatured proteins are usually less functional when used in food systems. It is not known, at least quantitatively, how egg-yolk protein denaturation or coagulation will affect PL extraction. Wu and Wang (2003) reported that oil contained in the raw material tended to give higher PL recovery.

The objectives of this research were:

1) To study the effect of protein denaturation on PL extraction and the feasibility of PL extraction without deoiling;
2) To develop a fractionation procedure that is feasible and environmental friendly; and
3) To characterize the important functional properties of the final lecithin product.

Thesis Organization

This thesis consists of a general introduction, followed by two research papers and a general conclusion section. The papers are in the required journal format for the American Oil Chemists' Society.

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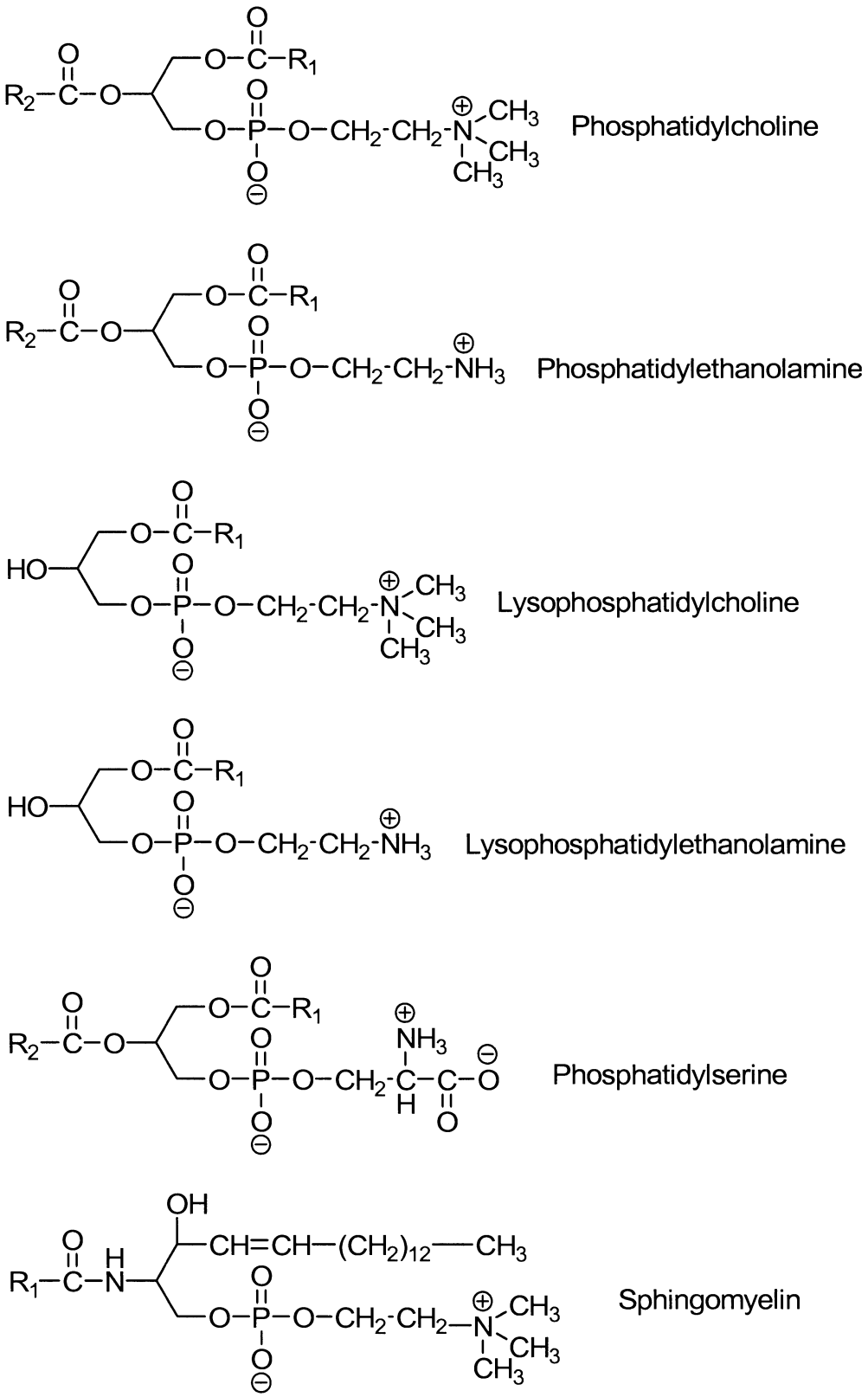

Figure 1. Structures of the main PLs present in egg-yolk lecithin.

CHAPTER 2. EFFECT OF PROTEIN DENATURATION AND OIL ON EGG-YOLK LECITHIN FRACTIONATION

Luz E. Palacios[1] and Tong Wang[1, 2]

Abstract

Egg-yolk lecithin has a unique composition and potential applications, but the information on extraction of lecithin or phospholipids (PLs) from egg yolk is limited. In this research, the effects of yolk oil and yolk protein denaturation on PL extraction or fractionation with ethanol were studied. Fractionation was performed with deoiled and undeoiled yolk and with heated and unheated yolks. Yield of extracted fraction relative to initial material, PL purity and cholesterol content of both PC-enriched and remaining PL fractions were determined. The yield and PL purity of PC-enriched fractions obtained from undeoiled yolk were 23.9 and 35.7%, and those obtained from deoiled yolk were 13.5 and 53.3%. The recovery of total PLs in two fractions was higher (70%) from the undeoiled than from the deoiled yolk (60%). However, protein denaturation only had slight effect on PL extraction. Better enrichment of PC was observed by extraction from undeoiled than from the deoiled yolk. Cholesterol content of the PC-enriched fraction obtained from the undeoiled yolk was much higher than that from the deoiled yolk.

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Key words: Cholesterol, egg yolk, fractionation, lecithin, phospholipids.

Introduction

Egg yolk typically contains 43.8% solids, of which 34.3% is protein, 55.8% is total lipid, 3.4% is ash, and 3.6% is carbohydrate (American Egg Board, 2002). Phospholipids (PLs) represent approximately 10% of the wet weight of egg yolk (Chi and Lin, 2002). The main components of egg-yolk lecithin are phosphatidylcholine (PC, 80.5%) and phosphatidylethanolamine (PE, 11.7%). The extraction of total lipids or the PLs from yolk is desirable because of the unique properties and valuable applications of these products (Sim, 1994). Egg-yolk PC is reported to significantly lower cholesterol absorption in rats compared with soybean PC (Jiang *et al.*, 2001). Egg-yolk lecithin contains relatively more saturated fatty acids than soybean lecithin does and it may have better oxidative stability. Antioxidant activity of egg-yolk PLs was reported in a recent study (Sugino *et al.*, 1997).

There is limited information on PL fractionation from egg yolk (Larsen and Froning, 1981; Tokarska and Clandinin, 1985; Sim, 1994 and 1995; Miyata and Matsumoto, 2001; Nielsen, 2001; Hruschka et al., 2003). The most common scheme of PL fractionation uses dried yolk and an initial deoiling step, and the PLs are extracted from the deoiled material with ethanol. Alternatively, yolk is extracted with ethanol first and then acetone is used to remove the oil from the ethanol extract. No quantitative comparison of these two different schemes, i.e. fractionation with and without oil, has been reported. From previous findings in our laboratory (Wu and Wang, 2003), oil contained in the raw material tended to give higher PL recovery. Dried yolk was typically used as starting material because of the belief that lipid and protein in fresh egg yolk are intimately associated and the lipids are not easily

extractable with non-polar solvent (Warren *et al.*, 1988). Yolk drying is energy intensive and the heat-denatured proteins are usually less functional when used in food systems. It is not known, at least quantitatively, how egg-yolk protein denaturation or coagulation will affect PL extraction. Extraction of lipids from raw and cooked egg have been both investigated, but a paired study is lacking. Therefore, the objectives of this experiment were to examine how egg-yolk protein denaturation affects PL extraction and if it is feasible and beneficial to extract PLs directly from the whole yolk without first deoiling.

Materials and Methods

Egg preparation. Grade A Monty eggs (Monty Produces, Monticello, IA) were purchased from a local grocery store. Ten eggs were heated in boiling water for 15 minutes, and the egg yolk was separated from the white. Another ten raw eggs were carefully broken to separate the yolk from the white and the combined yolk was kept in a cold room (5°C) before use. Moisture content of the heated and unheated egg yolks were determined by using a conventional oven drying method at 100°C for 4 hr.

Lecithin extraction from undeoiled egg yolk. The flow chart of lecithin extraction is outlined in Figure 1. Ethanol (100%) was added to approximately 5 g of heated or unheated egg yolk to a final of 5:1 ratio of solvent to egg yolk (wet weight). The mixture was stirred until egg yolk was completely dispersed. The final concentration of ethanol was 91% after it was diluted with water contained in the fresh yolk. The sample was then centrifuged at 400 x g for 5 minutes. The PC-enriched fraction (supernatant) was transferred to a previously weighed round bottom flask and the ethanol was removed by rotary evaporation. The dried PC-enriched fraction was weighed and transferred to a 15-mL vial with 10 mL of

chloroform/methanol (2:1, v:v) for HPLC analysis. The residual egg yolk was dried at room temperature for couple of days, and then it was deoiled with acetone using AOCS Official Method Ja 4-46 (1994). The acetone extract was kept for further quantification of the neutral oil. After acetone deoiling, 40 mL of chloroform/methanol (2:1, v:v) was used to extract residual lipids from the yolk. Water-saturated butanol also was used to extract any remaining polar lipid from the yolk residual. The combined lipids were washed using the Folch method (Folch *et al.*, 1957). This fraction was referred as remaining PL fraction.

Lecithin extraction from deoiled egg yolk. Acetone was used to deoil 5 g of egg yolk (heated or unheated) following the AOCS official method as above. The supernatant obtained after centrifugation was kept for quantification of the neutral oil. The precipitate was dried at room temperature for couple of days, then, aqueous ethanol at 91% concentration was used at a 5:1 ratio of solvent to egg yolk to extract PLs as described above. Extraction of the remaining PL fraction was also done with chloroform/methanol (2:1, v:v) and water-saturated butanol as outlined above.

The parameters measured were yield of the fraction, PL purity and recovery, and cholesterol content of both PC-enriched and remaining PL fractions. Yield was the percentage of fraction obtained relative to the total dry weight of the starting material. Purity was the percentage in a fraction of total PLs as quantified by HPLC. Recovery was the amount of total PLs in the fraction divided by the total PLs in the starting material. Cholesterol content was the percentage of cholesterol in the fraction, and it was measured by saponification and then GC quantification as described below.

Quantification of PLs by HPLC. A Shimadzu high performance liquid chromatography (HPLC) system with LC-600 liquid delivery module, silica column (250

mm length, 2.1 mm id, Alltech, Deerfield, IL), VAREX IIA evaporative light scattering detector (ELSD), and CR501 Chromatopac integrator was used to quantify PLs. An isocratic elution with chloroform/methanol/water/acetic acid (63.5:32:4:0.5, v/v) at flow rate of 0.5 mL/min was used. Nitrogen with a flow rate of 2.3 L/min was applied to evaporate the solvent in the 115° C drift tube of the ELSD. An injection loop with 20-μL volume was used. External standard curves were established to quantify PLs.

Cholesterol quantification by GC. About 100 mg of the lipid sample was accurately weighed and transferred into a glass vial. Two mL of 1 N potassium hydroxide (in 95% ethanol) was added to the sample, which was then placed in a boiling water bath for two hours. After the saponification reaction, 5 mL of distilled water was added to the mixture, and three 5 mL portions of diethyl ether were used to extract the unsaponifiable materials. Then, the ether extract was washed with distilled water and evaporated under nitrogen. The crude unsaponifiable matter was dissolved in one mL hexane containing cholestane as an internal standard, and duplicate samples were injected in a Hewlett Packard 5890 Series II GC system equipped with a fused silica capillary column (SAC-5, 30 M x 0.25 mm x 0.25-μm film thickness from Supelco, Bellefonte, PA). GC oven temperature was 285°C, while injector and detector temperatures were 300°C.

Initial composition of the egg yolk. To determine the composition profile of the initial yolk material, total lipid was extracted by chloroform:methanol (2:1, v:v) and water-saturated butanol. A Folch wash was applied to remove water-soluble contaminants. Neutral lipid was removed from the total lipid extracted by acetone, and PLs were quantified by HPLC. The total oil content was calculated as the oil obtained by acetone deoiling plus the non-PL portion of the deoiled PL fraction. The total PL content was the sum of the PLs in the

PL fraction and in the oil fraction. Protein content was determined after drying and weighing the residual solid.

Statistical analysis. Data were analyzed with the General Linear Model (GLM) of SAS program. Least significant difference (LSD) values were used to examine if there were significant treatment effects on PL extraction in this two-factor factorial treatment design: presence of oil (undeoiled and deoiled yolk) and protein denaturation (heated and unheated yolk).

Results and Discussion

The initial composition of the egg yolk was 51.6% moisture, 16% protein, 7% PLs (PC+PE), and 25.4% neutral lipids, which included 0.6% total cholesterol and 24.8% triacylglycerols. Therefore, on the based of total lipid, 78.4% was neutral lipids (including 1.8% cholesterol) and 21.6% was PLs. There is considerable variation of egg yolk composition as reported in the literature, possibly because of differences in breed and feed of the hens. Tokarska and Clandinin (1985) reported that lipid profile of egg yolk was 65% neutral oil, 28.3% PLs, and 5.2% cholesterol.

The results of lecithin fractionation and quantification are presented in Tables 1 and 2, and the statistical data are shown in Table 3. Moisture content in the heated egg yolk was 49.9%, which is reduced slightly compared with that of the unheated yolk.

Effect of oil and protein denaturation on yield of PL fractions. The status of yolk protein (unheated and heated) had no effect on yield of the PC-enriched and remaining PL fractions (Table 1). However, the yields of PC-enriched fraction were significantly different between the undeoiled and the deoiled egg yolk, which were 23.9 and 13.5%, respectively.

The yields of remaining PL fraction were 4.2 and 5.9% from the two yolk materials, and this difference is also statistically significant. The higher yield from the undeoiled material is because of the extraction of significant amount of oil into ethanol soluble fraction. Consequently, the purity of the PC-enriched fraction was much higher from the deoiled than from the undeoiled materials. Conventionally, heating or protein denaturation of the oil-bearing material is believed to facilitate lipid extraction through the disruption of protein and oil interaction and the coalescence of the oil droplets. However, the data shown here suggests that heating is unnecessary for PL extraction with ethanol, possibly, because ethanol is a strong polar solvent and it can disrupt the protein-lipid interaction in the yolk lipoprotein.

Effect of oil and protein denaturation on the purity and recovery of PL fraction. Protein denaturation did not affect purity of the PC-enriched fraction, but it significantly affected purity of the remaining PL fraction (Tables 1 and 3). When protein was denatured, the extraction of PLs in the remaining PL fraction from the yolk was more complete than when the protein was not denatured, regardless of whether the oil was removed before or after the ethanol extraction. The presence of oil significantly decreased the purity of PC-enriched fraction, with a PC-enriched fraction purity of 35.7% for the undeoiled yolk and 53.3% from the deoiled yolk (Tables 1 and 3). When acetone deoiling was done after ethanol extraction, the remaining PL fraction was purer than when acetone deoiling was done before the ethanol extraction (Table 1). The purity or PL content in these products is low because of the large amount of oil co-extracted with PLs. Other impurities could be minor polar lipids that were not quantified in the HPLC analysis. Additional deoiling/purification with acetone may have to be used to further increase the purity. Alternatively, if acetone is undesirable in

PL production because of environmental or safety concerns, multiple extractions with ethanol will improve the purity (Wu and Wang, 2003).

The PL recovery in the PC-enriched fraction was not affected by protein denaturation, but again, protein denaturation significantly increased PL recovery in the remaining PL fraction, with 9.1 and 8.1% recovery from the heated and unheated materials (mean of the values for deoiled and undeoiled, Tables 1 and 3). Statistically, the presence of oil did not affect PL recovery in PC-enriched fraction. However, there was a clear trend that the total PL recovery in the PC-enriched fraction from the undeoiled yolk was much higher than that from the deoiled yolk (60.0 and 50.6%). This may be caused by the loss of PLs during acetone deoiling before ethanol extraction. The water in the fresh yolk may be partially dissolved in the acetone, making acetone polar, and subsequently the solubility of PLs in such acetone-water mixture may be increased. Wu and Wang (2003) also observed this phenomenon. A total of 30-40% PLs was lost during the fractionation process in our research.

The highest PL purity obtained in our experiment was 55% from the deoiled and heated egg yolk. However, Sim (1994) reported a PL product with 89% purity prepared from spray-dried yolk when the material was extracted with aqueous ethanol. Sim's report offers no explanation to account for such different results from ours. There might be considerable differences in the physico-chemical properties of the spray-dried and heat-denatured yolk that could have resulted in the difference in PL extraction.

PL class composition as affected by oil and protein denaturation. Protein denaturation significantly affected PL class composition of the PC-enriched fraction obtained from the deoiled material, but not from the undeoiled material (Tables 2 and 3). When yolk

protein was denatured, the extraction of PC with ethanol from the deoiled yolk was more effective than from the undeoiled yolk.

In the presence of oil, the extraction of PC with ethanol was more effective than in the deoiled material, as shown by higher percentage of PC (72 and 68%, mean of values of the heated and unheated samples, Table 2). Although these differences were not great, they were statistically significant. This observation could be the result of the oil acting as a solvent for PE, making PC relatively more extractable with ethanol.

For the remaining PL fraction, heating did not affect PL class composition of the fraction obtained from undeoiled yolk. However, from the deoiled yolk, PC percentage was significantly lower from the heated yolk than from the unheated yolk. Possibly, the PC was already preferentially extracted by ethanol from the deoiled yolk. The remaining PL fraction from the undeoiled material had higher PC percentage than that from deoiled material (Table 2). Schneider (1989) reported that alcohol fractionated egg-yolk PLs contained 69% PC and 24% PE, which is in generally agreement with our results for the PC-enriched fractions as shown in Table 2.

Overall, extraction of PLs from undeoiled egg yolk resulted in relatively higher PL recovery and higher PC percentage in both the PC-enriched fraction and the remaining PL fractions than from the deoiled yolk.

Cholesterol content in fractionated lecithin. There were significant differences in cholesterol content in the two PL fractions obtained from the undeoiled and deoiled yolk (Tables 1 and 3). Deoiling the egg yolk before ethanol extraction was beneficial in removing cholesterol from the PL products. Evidently, cholesterol is very extractable with ethanol if it is present in the initial material. Deoiling with acetone can extract all triacylglycerols and

cholesterol, as well as 15% of the PLs according to Nielsen (2001). The PC-enriched fraction from undeoiled egg yolk had increased concentrations of cholesterol (3.8%, Table 1) compared with 1.9% cholesterol in total egg yolk lipids.

Calculated oxidizability of the PL products. As mentioned in the introduction, egg-yolk PLs are more saturated, and they should be more oxidatively stable than soybean lecithin. This can be a significant advantage of egg-yolk lecithin over soy lecithin, because in certain food preparations, particularly in emulsions, off-flavor generation has been attributed to lecithin oxidation. Oxidizability (Fatemi and Hammond, 1980) based on fatty acids composition was calculated for both soybean and egg-yolk lecithin using PL fatty acid composition reported by Wang et al. (1997) and our GC data, and PL class composition as determined by HPLC. The calculated oxidizability of soy lecithin is 0.76, which is much higher than the value for egg lecithin of 0.49. Therefore, egg-yolk lecithin may have higher oxidative stability than soybean lecithin.

Conclusions

Extraction of PLs from fresh egg yolk with ethanol is feasible. Although, oil contained in the yolk was partially extracted with ethanol, decreasing PL purity of the PC-enriched fraction, it also facilitated PC enrichment. Egg-yolk protein denaturation was not necessary for the initial PL extraction with ethanol; however, it made the subsequent extraction of the remaining PL fraction slightly more efficient. This comparative study provides the basis for developing a more efficient PL fractionation procedure from egg yolk.

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Table 1. Mean value and standard deviation of yield, purity, recovery, and cholesterol content of fractionated egg-yolk lecithin

		PC-Enriched Fraction		Remaining PL Fraction	
	Yolk Treatment	Undeoiled	Deoiled	Undeoiled	Deoiled
Yield, %	Heated	24.4±0.1	13.3±0.4	4.0±0.0	5.6±0.4
	Unheated	23.4±0.0	13.7±0.0	4.3±0.1	6.1±0.6
Purity, %	Heated	34.9±1.5	55.0±2.2	32.3±2.6	22.2±1.9
	Unheated	36.4±7.2	51.6±0.2	28.4±0.8	18.6±1.5
Recovery, %	Heated	60.9±3.6	52.3±3.5	9.2±0.9	8.9±0.1
	Unheated	59.1±11.9	48.8±1.3	8.4±0.0	7.9±0.1
Cholesterol, %	Heated	3.8±0.2	0.4±0.0	0.2±0.1	0.3±0.0
	Unheated	3.7±0.3	0.4±0.1	0.1±0.1	0.3±0.0

Yield = 100 x quantity of PL fraction / quantity of initial yolk material;
Purity = 100 x total PLs as quantified by HPLC / quantity of PL fraction;
Recovery = 100 x total PLs as quantified by HPLC / total PLs in the initial yolk material.
n=2, two replicates of egg yolk fractionation

Table 2. Phospholipid class composition (%) of fractionated egg-yolk lecithin

	PC-Enriched Fraction			
	PC		PE	
Yolk Treatment	Undeoiled	Deoiled	Undeoiled	Deoiled
Heated	72.3±0.2	69.9±0.9	27.7±0.2	30.1±0.9
Unheated	72.0±0.8	66.5±0.2	28.0±0.8	33.5±0.2
	Remaining PL Fraction			
	PC		PE	
Yolk Treatment	Undeoiled	Deoiled	Undeoiled	Deoiled
Heated	52.8±0.5	37.0±2.2	47.2±0.5	63.1±2.2
Unheated	52.5±3.2	45.0±2.4	47.5±3.2	55.0±2.4

PC, phosphatidylcholine; PE, phosphatidylethanolamine.

Table 3. Probability (P) and $LSD_{0.05}$ values for the effect of protein denaturation and oil on the fractionation of egg-yolk lecithin

	PC-Enriched Fraction					
	Yield	Purity	Recovery	PC	PE	Cholesterol
P (Heat)	0.3009	0.7440	0.6046	0.0113	0.0113	0.9177
P (Oil)	<0.0001	0.0029	0.1094	0.0007	0.0007	<0.0001
P (Heat x Oil)	0.0321	0.4116	0.8607	0.0201	0.0201	0.6347
$LSD_{0.05}$	0.58	7.56	12.76	1.16	1.16	0.38
	Remaining PL Fraction					
	Yield	Purity	Recovery	PC	PE	Cholesterol
P (Heat)	0.1761	0.0433	0.0399	0.0745	0.0745	0.5582
P (Oil)	0.0023	0.0016	0.2547	0.0019	0.0019	0.0156
P (Heat x Oil)	0.6343	0.9115	0.8060	0.0623	0.0623	0.0929
$LSD_{0.05}$	0.70	3.58	0.85	4.48	4.48	0.10

x Indicate interaction.

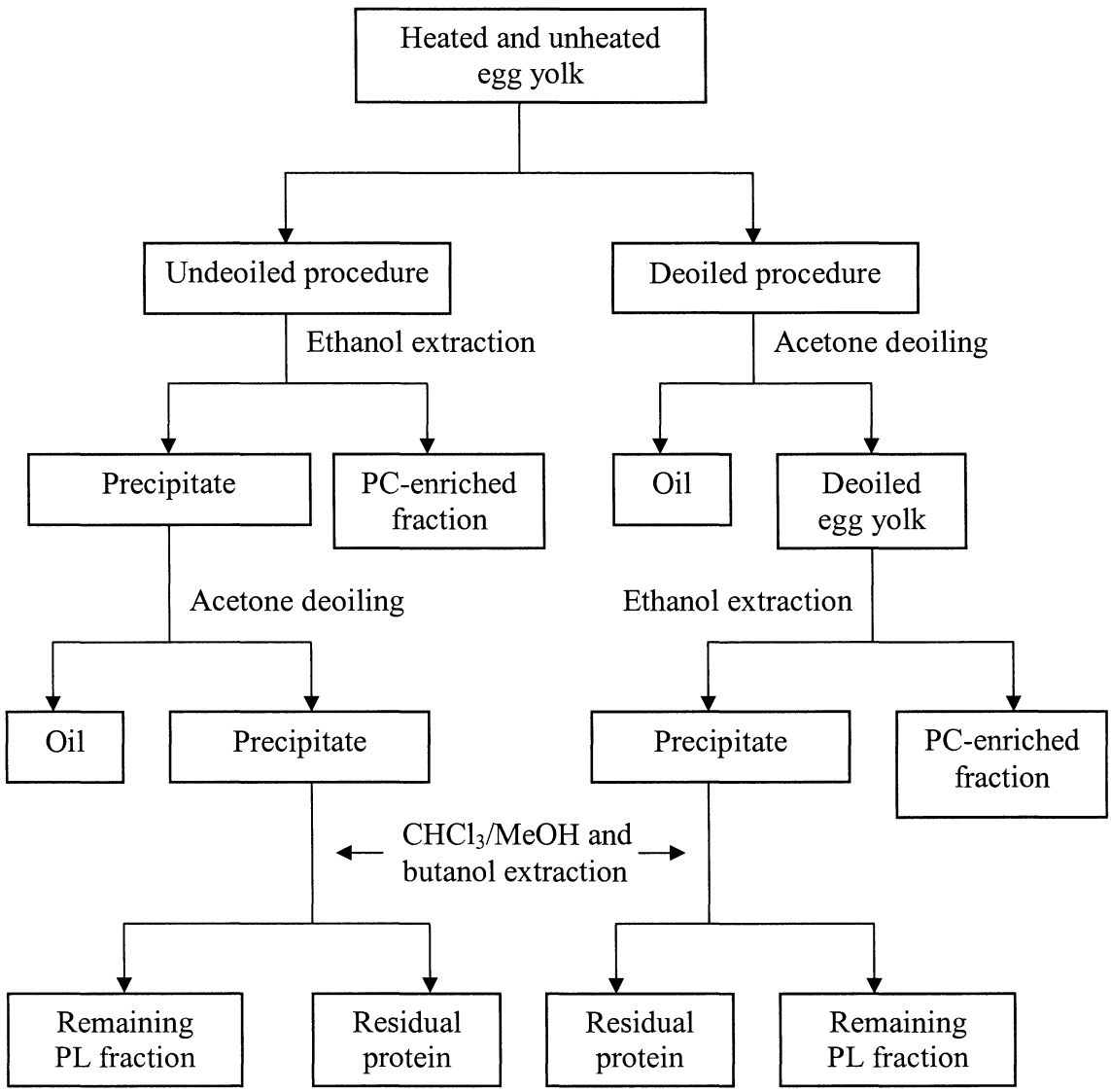

Figure 1. Fractionation of heated and unheated egg yolk by undeoiled and deoiled procedures

CHAPTER 3. EGG-YOLK LECITHIN FRACTIONATION AND CHARACTERIZATION

Luz E. Palacios[1] and Tong Wang[1, 2]

Abstract

Egg-yolk lecithin has phospholipid class and fatty acid compositions that are different from soybean lecithin, and egg-yolk lecithin may have unique functional properties. The purposes of this research were to develop an efficient method for the fractionation of large amount of egg-yolk lecithin from fresh egg yolk, and to evaluate its functional properties. Ethanol was used to dehydrate and partially extract the phospholipids (PLs), followed by hexane to extract the total lipids. An acetone precipitation of PLs from the final polar lipid fraction was necessary to remove residual neutral lipids, especially cholesterol. Reduction of solvent used in the large scale process resulted in a slightly but statistically significantly lower lecithin yield compared with the analytical scale. PL purities for the analytical and large scales were 95.9±0.3 and 94.9±0.1%, respectively. Surface tension reduction, emulsion stability and oxidative stability studies were conducted to characterize egg-yolk lecithin's functional properties. Soybean lecithin and egg-yolk lecithin had similar surface activity as evaluated by surface tension reduction in an aqueous system and the critical micelle concentration (CMC). Soybean lecithin created a more stable emulsion than did egg-yolk lecithin. However, egg-yolk lecithin was more oxidatively stable than soybean lecithin.

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Key words: egg-yolk lecithin, emulsion stability, functionality, phospholipids, oxidative stability, surface tension.

Introduction

Egg yolk is a good source of phospholipids (PLs) representing approximately 10% of the wet weight of egg yolk (Chi and Lin, 2002), which is equivalent to about 22% of the total egg yolk solid. The main components of egg-yolk lecithin are phosphatidylcholine (PC, 80.5%) and phosphatidylethanolamine (PE, 11.7%). Egg yolk also contains lysophosphatidylcholine (LPC), sphingomyelin (SM), and neutral lipids in minor quantities. The extraction of the total lipids or the PLs from yolk is desirable because of the unique properties and valuable applications of these products (Sim, 1994). The oil is used in nutritional supplements, baby foods, and for parenteral feeding because of its relatively high ω-3 fatty acid content. The PLs are used in pharmaceutical and cosmetic industries as an emulsifier. It is reported that dietary egg-yolk PC can significantly lower cholesterol absorption in rats compared with soybean PC (Jiang *et al.*, 2001). Egg-yolk lecithin contains relatively more saturated fatty acids than soybean lecithin does and it may have better oxidative stability than soybean lecithin.

According to limited information in the literature, egg-yolk lecithin is primarily extracted with solvents such as diethyl ether, hexane, chloroform, and ethanol. However, some of these solvents are considered undesirable because of environmental and health concerns (Sim, 1994; Nielsen, 2001). Earlier researches mainly focused on total lipid extraction and cholesterol removal. Sequential extractions with various solvents or multi-solvent system were investigated (Larsen and Froning, 1981; Tokarska and Clandinin, 1985).

Other PL fractionation techniques, such as ethanol extraction, low temperature crystallization to remove the solidified neutral oil (Sim, 1995), and ultrafiltration to isolate egg PLs (Miyata and Matsumoto, 2001) have been investigated. Hruschka *et al.* (2003) described a procedure of using low and high concentrations of aqueous ethanol in several steps to extract yolk PLs and they achieved 47% PL purity.

The most common scheme of PL fractionation uses dried yolk and an initial deoiling step. The PLs are extracted from the deoiled material with ethanol. Dried yolk was used as starting material because of a general belief that lipid and protein in fresh yolk are intimately associated and the lipids are not easily extractable with non-polar solvent (Warren *et al.*, 1988). Yolk drying is energy intensive and if the proteins are heat-denatured, they are usually less functional when used in food systems.

A rapid and economical method to produce yolk oil and lecithin directly from fresh yolk without using hazardous solvent is still needed. The objectives of this research were to develop a method for the fractionation of egg-yolk lecithin and to compare its functional properties with those of soybean lecithin.

Material and Methods

Egg yolk preparation and chemicals. Fresh farm eggs distributed by Boomsma's Inc. (Alden, IA) were purchased from a local grocery store. Eggshells were carefully broken and yolk was separated from the egg white, and the egg yolk was stored in a refrigerator (5°C) until use. Moisture content of the combined egg yolk was determined in an oven at 100°C for 4 hours. The solvents and chemicals used in the PL extraction, PL quantification, and

cholesterol determination were aqueous ethanol (87, 90, 95 and 100%), hexane, acetone, chloroform, methanol, diethyl ether, ammonium hydroxide, and 1 N potassium hydroxide (in 95% aqueous ethanol).

Analytical-scale lecithin extraction and fractionation by solvent partition. The multiple-step procedure of lecithin extraction from fresh egg yolk is outlined in Figure 1. For the initial extraction, 100 mL ethanol (95%) was added to 30 g of fresh egg yolk in a 200-mL centrifuge bottle and stirred until the egg yolk was completely dispersed. Then, the mixture was centrifuged at 1900 x g for 5 minutes, and the supernatant containing water, some polar and neutral lipids was transferred to a separatory funnel. Neutral lipids from the precipitate were extracted twice with 50 mL of hexane. The hexane extracts were transferred to the same separatory funnel. The protein precipitate was extracted two times with 50 mL ethanol (95%) to remove any residual polar lipids. These ethanol extracts were combined with the ethanol and hexane extracts in the separatory funnel. The separatory funnel was then thoroughly but gently mixed and left to sit for one hour for phase separation. The ethanol phase was removed and the hexane phase was mixed with additional 50 mL ethanol (90%) and left for phase separation. Hexane was removed by rotary evaporation, leaving behind the neutral lipids (NL-1) which were determined gravimetrically. The ethanol phase was combined with the previous ethanol phase, and the solvent was evaporated. The remaining polar lipid material was dissolved in 35 mL of hexane and transferred to a 200-mL centrifuge bottle where 150 mL of chilled acetone (4°C) was added and carefully stirred to precipitate the PLs. Then, the centrifuge bottle was placed in an ice-water bath for 15 minutes and centrifuged at 1500 x g. The supernatant was removed and solvent was evaporated by rotary evaporation.

This fraction should contain any neutral oil and cholesterol that tends to be readily extractable into ethanol, and it was named NL-2. The precipitate was the purified PLs.

Quantification of PLs by HPLC. Quantification of PLs in samples was done using a Beckman-Coulter (Fullerton, CA) high performance liquid chromatography (HPLC) system equipped with auto sampler 508, solvent delivery module 126, diol normal-phase silica column (250 mm x 4.6 mm i.d., with integral guard column, Advanced Separations Technologies, Whippany, NJ), and an evaporative light scattering detector (ELSD 2000; Alltech, Deerfield, IL). A gradient program with two mobile phases at a flow rate of 1 mL/min was used: A was chloroform/methanol/ammonium hydroxide (80:19:1, v/v), and B was chloroform/methanol/water/ammonium hydroxide (50:48:1:1, v/v). The gradient program used is shown in Figure 2. Nitrogen with a flow rate of 1.7 L/min was used to evaporate solvent in draft tube at 60°C. Standard curves for the two major PL classes in egg-yolk lecithin, i.e., PE and PC, were established.

Characterization of PLs by TLC and GC. The egg-yolk PL sample was dissolved with chloroform:methanol (2:1, v:v) in a 10-mL volumetric flask. Then, 0.15 mL of the solution was streaked on a 20 x 20 cm, 500-μ thick Adsorbsil-plus 1 preparative silica plate (Alltech), and the plate was developed with chloroform/methanol/acetic acid/water (100:45:5:2, v:v:v:v). Bands were visualized with of 2',7'-dichlorofluorescein (0.1% in methanol) spray and viewed under UV light. PC and PE bands were collected and transferred to clean vials in which the internal standard methyl heptadecanate (17:0) in hexane was previously transferred and the solvent evaporated under nitrogen. Approximately 1.5 mL of 0.5 M sodium methoxide was added, an amount sufficient to cover all the silica in the vials. The transesterification reaction was allowed for 40 minutes at 50°C. Distilled water (a few

drops) was added to stop the reaction, and 1.0 mL hexane was used to extract the fatty acid methyl esters (FAME). About 1.0 μL of each extract was injected into a Hewlett-Packard (HP) 5890 Series II gas chromatography (GC) system with capillary column (SP-2330, 15 m x 0.25 nm x 0.2-μm film thickness from Supelco, Bellefonte, PA) for fatty acid composition determination. GC oven temperature was 190°C, while injector and detector temperatures were 230°C.

Cholesterol quantification. Cholesterol determination begun with the accurate weighing of about 100 mg of each lipid sample into a glass vial. Two mL of 1 N potassium hydroxide (in 95% ethanol) was added to each sample, which was then placed in a boiling water bath for two hours. After the saponification reaction, 5 mL of distilled water was added to the mixture, and three 5 mL portions of diethyl ether were used to the extract unsaponifiable materials. The ether extract was washed with distilled water, and the ether was evaporated under nitrogen. The dried unsaponifiable matter was dissolved in 1 mL hexane containing a known amount of cholestane as internal standard. Duplicate samples were injected in a Hewlett Packard 5890 Series II GC system with fused silica capillary column (SAC-5, 30 m x 0.25 mm x 0.25-μm film thickness from Supelco, Bellefonte, PA). GC oven temperature was 285°C, while injector and detector temperatures were 300°C.

The parameters used to evaluate the efficiency of the extraction were yield, cholesterol content, and PL distribution for fractions NL-1, NL-2, and PLs, and purity of PL fraction. Yield was the amount of fraction obtained divided by the total starting dry weight. Cholesterol content was expressed as weight percentage of cholesterol in the fraction. PL distribution was the amount of PLs in the fraction divided by the total PLs in the starting

material. Purity was the weight percentage of total PLs as quantified by HPLC in the fraction.

Large-scale lecithin fractionation for functionality evaluation. A large quantity of lecithin was needed for functionality studies. Changes in the analytical fractionation procedure were necessary to ensure feasibility of the scaled-up processing. There were three modifications. First, the ratio of egg yolk and total solvent was reduced from 1:16 in the analytical scale to 1:9.5 in the large scale. Second, the ethanol concentration was changed. An early research of polar lipid isolation (Galanos and Kapoulas, 1962) demonstrated a nearly complete recovery of polar lipid when 87% aqueous ethanol was used in a petroleum ether-alcohol solvent partition of neutral and polar lipids. Therefore, we decided to use 100% and 87% ethanol instead of 95% and 90% at two steps of lipid extraction. The moisture contained in the egg yolk would provide the right amount of water to give the ethanol a final concentration of 87%. The third modification was the number of extractions in each stage. The fact that the amount of solvent needed to be reduced required the use of multiple times (three times) of extraction in each step to ensure extraction efficiency. Three replicates were conducted in the production of this lecithin. Parameters measured and calculated were the same as for the analytical procedure.

Surface tension reduction of egg-yolk lecithin. The PL fractions from the large scale lecithin extraction were dispersed in distilled water at a high concentration and then diluted in series to obtain lower concentrations. Soy lecithin (Fisher Scientific, Pittsburg, PA, 99% purity) was used as a control. Surface tension of the aqueous dispersion was determined at each concentration with a FACE Automatic Surface Tensiometer (Model CBVP-Z, Tantec

Inc., Schaumburg, IL). The surface tension was plotted against the logarithm of the concentration to determine critical micelle concentration (CMC) of the PLs.

Emulsion stability. Egg-yolk PL fractions and soybean lecithin were used as emulsifier to make oil in water (o/w) emulsions. The proportion of oil to water was 2:8 (v:v), whereas the emulsifier concentrations used were 5% and 10% of the oil weight. Emulsifier was dispersed in distilled water to facilitate its incorporation in the o/w emulsion. A stock solution (100 mg/mL) of each emulsifier was prepared. Water, emulsifier, and oil were measured and blended with a Hamilton Beach® blender (Model 51101) for 3 minutes. Then, emulsions were transferred to 50-mL graduated cylinders, and the volume of separated discontinuous phase (oil) was recorded periodically.

Oxidative stability of egg-yolk lecithin. A 20 mg/mL lecithin solution in hexane was prepared for each egg-yolk PL fraction as well as for the control soybean lecithin. To ensure an even and maximal exposure of the lecithin samples to oxygen, a modification of the process used by Wu and Wang (2003) was made. Instead of using mineral oil as dispersing vehicle, glass beads (5 mm diameter) were used. A fixed number of glass beads were placed in 9 labeled 20-mL vials. Then, 0.3 mL (6 mg) of the stock lecithin solution was dispensed into each vial. The vials were shaken to allow the coating of the glass beads with lecithin solution. The hexane was then removed by blowing nitrogen into the vial. The vials were then placed in a conventional oven at 55°C for 16 days with the removal of one vial every 48 hours. At the end of this term, peroxide quantification was done by a modified procedure of the ferric thiocyanate method (Driver, 1963). Hydroperoxides in the samples were quantified by measuring the absorbance at 515 nm. Oxidized soybean oil was used to establish a standard curve as following: first, the actual peroxide value of the oxidized soybean oil was

determined by the standard iodometric method (AOCS, 1994), then, a stock solution of the oxidized soybean oil was prepared by dissolving known amount in ethanol-benzene (80:20). Different amounts of this solution were placed in a series of 10-mL volumetric flasks and color developed as before. A linear standard curve was obtained when plotting the absorbance versus the micro-equivalent (μeq) of peroxide present.

Results and Discussion

Lecithin fractionation. The moisture content of the fresh egg yolk was 49.4%. Yield percentages for each of the main fractions from the analytical and large scales are shown in Table 1. Reproducibility of the treatments is good, as indicated by the relatively small standard deviations. However, the yield differences between the two scales are statistically significant, although not considerable, possibly because of the smaller volume of solvent used in the large scale processing. The NL-1 yield of the large scale (21.7±0.1%) was significant higher than from the analytical scale (19.9±0.7%). This could be because of the insufficient separation of polar lipids from the neutral lipids. The yield of NL-2 fractions from final acetone precipitation of PLs and removal of neutral lipid also indicated lower efficiency of the large scale compared with the analytical scale processing. However, significant amounts of solvents were saved by this lower solvent to egg yolk ratio.

On the dry weight basis, our yolk material contained 17.7% protein, 11.0% PLs (based on 95.4% purity), and 22.5% neutral oil. These values are similar to the values reported by the American Egg Board, except our total lipid content (66% of the dry weight) was higher than the reported 55.8%. We believe this difference is possibly because of variation in breed and feed of the hens.

PL quantification by HPLC. The use of a new HPLC column specific for PL characterization (different from that used in chapter 2) reduced equilibration time between runs, and sharper peaks were also obtained. PE and PC standard curves were established by injecting different concentrations of a PE and PC mixture (15% PE and 85% PC) into the HPLC. An individual calibration equation was obtained for each standard by plotting the peak area versus the amount of sample injected. The standard calibration equation for PE was Area = 125,160 x $(\text{Amount})^{1.61}$ (R^2=0.999) and that for PC was Area = 12,146 x $(\text{Amount})^{2.05}$ (R^2=0.995). Vegetable oil and cholesterol standards were also injected to identify each peak on the chromatogram (Figure 3). The first peak was identified as neutral oil, and the second peak was cholesterol. PL purity for the analytical scale is slightly, but statistically significantly higher (95.9±0.3%) than the purity of the large scale (94.9±0.1%), as seen in Table 2. Overall, this new fractionation method for egg-yolk lecithin is much more efficient and effective than the method of others. For example, the Hruschka's patented procedure (2003) in which low concentration ethanol was used to fractionate oil and polar lipids resulted in 47% purity of the PL fraction.

The PL content and distribution in each of the main fractions from the analytical and large scale extractions are shown in Table 3. There were statistically less PLs lost in the NL-2 fraction of the large scale than did in the NL-2 of the analytical scale. It seemed that reduction of acetone used for precipitation reduced PL loss. PL distribution in the neutral and PL fractions of both scales was about 4 and 96%, respectively. Therefore, the total PL recovery achieved by this fractionation procedure was exceptional.

Identification of plasmalogen by TLC and GC. Our HPLC analysis with the new column showed that the non-PL materials were cholesterol and neutral oil, and they were

present at about 5%. However, our earlier HPLC analysis with silica column showed a much lower PL content because of a wide peak that gave inaccurate peak integration and poor reproducibility. The presence of other compound in the PL fraction was tested by using TLC, no other major unknown was identified. However, when the TLC plate was developed three times, the PE band split into two bands. We were able to identify one of the bands as PE plasmalogen by a simple acid hydrolysis test (Christie, 1982). Plasmalogen is a vinyl ether phospholipid (Figure 4). The sn-1 position of glycerol is linked to the carbon chain by a vinyl ether bond instead of an ester bond. After acid hydrolysis, this vinyl ether bond is broken, forming an aldehyde. The acid hydrolysis products of the two separated PE bands were streaked on a 2 x 4 cm, 250-μm thickness, silica gel plate (Sigma-Aldrich, St. Louis MO). The plate was developed in hexane:diethyl ether (90:10, v:v) and then visualized with a potassium permanganate stain that is a mixture of potassium permanganate (1%) and potassium carbonate (6.7%) dispersed in 5% aqueous sodium hydroxide and water. Standard aldehydes (with C_8 and C_{14} chain length) were used as references on the TLC plate. It was shown that two aldehydes having a chain length of approximately C_{16} and C_{18} were present in one of the two samples. This PE plasmalogen band (moved higher on TLC plate than the PE band) was also transesterified and the fatty acid methyl esters were quantified. The quantity of plasmalogen was then calculated assuming the carbon chain length linking by the ether bond was C_{16}. The plasmalogen content was estimated at about 3.6% of the total yolk PLs. Rhodes (1957) reported that there is 0.9 % plasmalogen in yolk. More work is needed to fully characterize and quantify this unique lipid in egg yolk. Recent studies showed that plasmalogen prevented cholesterol oxidation in membranes (Maeba and Ueta, 2003; Zommara *et al.*, 1995).

The fatty acid composition of PE and PC of egg-yolk lecithin was determined by GC, as shown in Table 4. There are more saturated fatty acids in egg-yolk lecithin than in soybean lecithin. The oxidative stability of a lipid may be predicted by calculating its oxidizability using the fatty acid composition (Fatemi and Hammond, 1980). The oxidizability of the egg-yolk and soybean lecithin was calculated as 0.76 and 0.50, respectively.

Cholesterol content in fractionated lecithin. Cholesterol content in the PL fraction from the analytical scale was lower than those in the NL-1 and NL-2 fractions were (Table 5). From previous research, we found that cholesterol was very extractable with ethanol. It is evident that the last step in the procedure, i.e. acetone precipitation of the PLs, was necessary to remove the cholesterol from the PL fraction. It is obvious that the efficiency of cholesterol removal in the large scale process was not as high as in the analytical scale. This might be because of the reduction in solvent use in the purification step especially the last acetone precipitation step. Multiple acetone precipitations of the PLs maybe used to increase neutral lipid removal. In the analytical scale separation, 35.2% cholesterol was partitioned in the neutral oil fraction (NL-1), and 52.2% was removed during the final PL precipitation into NL-2. About 12.6% of the total cholesterol was retained in the lecithin product. In the large scale, processing, similar percentage of cholesterol (34.3%) was in the NL-1 product; however, the PL precipitation step removed much less cholesterol (28.3%) than that in the analytical scale, and much higher proportion of cholesterol (37.4%) went in the final lecithin product. The total cholesterol content in the initial material for the large scale processing was higher than that of the analytical scale, possibly because of different batches of egg being used.

Reduction of surface tension by egg-yolk lecithin in aqueous system. Measuring surface tension reduction and critical micelle concentration (CMC) are common ways of evaluating quality of emulsifiers or surfactants. Surface tension reduction of egg-yolk and soybean lecithin is shown in Figure 5. PL fractions and soy lecithin showed similar patterns. The surface tension was reduced as the concentration of the PL samples increased, and was lowered to a minimal value and then became independent of concentrations. The curve from each sample was divided into two parts: one part was the near-linear reduction of surface tension with concentration and the other part was the last few points when the surface tension was relatively unchanged. A linear trend for each portion was determined, and the concentration at which these two lines intercepted was determined as the CMC. The mean of the CMC values for the egg-yolk PL fractions was 15.3 mg/mL and that for soybean lecithin was 15.8 mg/mL. This CMC value for soybean lecithin was slightly different from the one reported by Wu and Wang (2003) where the CMC for the same brand soybean lecithin was 13.6 mg/mL. Surface tension was reduced to a mean of 35.1 mN/m for the yolk PL fractions and 30.2 mN/m for the soybean lecithin. These results suggest that egg-yolk lecithin has a surface activity similar to soybean lecithin. Some of the data point fluctuation in the graph could be because of minor defects and the cleanliness on the platinum plate used with the surface tensiometer.

Emulsion stability. The emulsions created with egg-yolk PL fractions broke faster than the emulsion created with the soybean lecithin (Figure 6). The three o/w emulsions containing 5% egg-yolk lecithin samples showed a similar trend. They had about 13% oil separation at 240 minutes, while the soybean lecithin showed only 8% oil separation. A peculiar change was observed in the o/w emulsions containing 10% of the emulsifiers. It is

typically believed that the more emulsifier we use, the more stable the emulsion will be. However, at 10% concentration, the percentage oil separated was higher (about 20-21% at 240 minutes) than for the lower concentration (5%). The emulsion containing 10% soybean lecithin had lower oil separation than at the 5% concentration. We believe that this maybe because of the difference of these two lecithin products in their PL class and fatty acid compositions. Soybean lecithin contains phosphatidylinositol (PI, 21%), whereas egg-yolk lecithin does not. However, egg-yolk lecithin is rich in PC, and it should be a good o/w emulsifier. An optimal combination of various types of PLs may be needed for an effective emulsifier. More research should be conducted to compare these two lecithins when used at concentrations lower than 5%. It is clear that soybean lecithin performed much better than did the egg-yolk lecithin in creating a stable o/w emulsion at the concentration studied or tested.

Oxidative stability of egg-yolk lecithin. Soybean lecithin was precipitated with acetone to remove any tocopherols that might be present, and thus eliminate the effect of antioxidants on oxidation. Figure 7 shows lipid hydroperoxide generation and degradation with time. The starting peroxide values (PV) for all samples were relatively high (15-20 meq/kg), indicating that oxidation had occurred during the fractionation procedure or storage. Egg-yolk lecithins oxidized to a maximal PV value of 24 to 27 meq/kg at about 47 hours, whereas the highest PV for soybean lecithin was 46 meq/kg at about 96 hours. The PV declined because of peroxide decomposition and termination of the free radical chain reaction. A soybean oil was also oxidized and peroxide quantified under the identical conditions. It reached a maximal PV of about 400 meq/kg at 290 hours before declining. Therefore, hydroperoxides of PLs seem to decompose much more readily than those of the

neutral oil did. The lower degree of oxidation of egg-yolk lecithin than soybean lecithin may be explained by its high content of saturated fatty acids. The relatively high content of unsaturated fatty acids in soybean lecithin (Table 4) caused it to form more hydroperoxides and will probably cause a stronger rancid flavor when used in foods.

The method established in this research for studying the oxidative stability of lecithin is much more effective than the method used previously by Wu and Wang (2003), in which, lecithin was dispersed in an inert mineral oil. No increase of peroxide value was obtained in that study, possibly because of the limited exposure of PLs to oxygen.

The oxidative stability of lecithin has not been studied as for other neutral lipids. There are a few studies on analytical procedures on hydroperoxide formation of PLs (Spickett *et al.*, 2001), however, the relatively stability of PLs from different sources and with different fatty acid composition has not been reported.

Conclusions

The procedures established in this research were successful and effective in producing a high purity egg-yolk lecithin. The last purification step of the PL fractionation with hexane and acetone not only removed neutral oil but also decreased the amount of cholesterol that was co-extracted with the polar lipids by ethanol. The quantitative method developed to study the oxidative stability of lecithins was successfully used in the study of PL oxidation. Egg-yolk lecithin proved to have a better oxidative stability than did soybean lecithin. The two types of lecithin had similar surface activity; however, soybean lecithin created a more stable emulsion than did egg-yolk lecithin.

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Table 1. Yield of main fractions from analytical and large scales of PL fractionation from fresh egg yolk

Fractionation		NL-1	NL-2	PLs	Protein
Analytical Scale (n=2)	Yield from fresh egg yolk (15.3 g, dry weight)	6.0±0.2	0.6±0.6	3.6±0.0	5.4±0.1
	Yield % from fresh egg yolk (30 g)	19.9±0.7 [b]	2.0±0.1 [a]	11.9±0.0 [a]	18.1±0.3 [a]
Large Scale (n=3)	Yield from fresh egg yolk (91.8 g, dry weight)	39.0±0.2	2.6±0.1	20.0±0.4	31.1±0.3
	Yield % from fresh egg yolk (180 g)	21.7±0.1 [a]	1.4±0.1 [b]	11.2±0.2 [b]	17.3±0.1 [b]
$LSD_{0.05}$		1.2	0.2	0.5	0.6

Yield = 100 x quantity of PL fraction / quantity of initial yolk material.
Abbreviations: NL-1, main neutral lipid fraction; NL-2, high cholesterol neutral lipid fraction; PLs, phospholipid fraction.
Same letter in the same column indicates that is not significant difference between the two scales of processing.
$LSD_{0.05}$: Least significant difference at P=0.05.
n, number of replicates in each scale

Table 2. Phospholipid purity and class composition of fractionated egg-yolk lecithin

Fractionation	Purity %	PE %	PC %
Analytical Scale (n=2)	95.9±0.3 [a]	19.1±1.1 [a]	80.9±1.1 [a]
Large Scale (n=3)	94.9±0.1 [b]	17.9±1.1 [a]	82.1±1.1 [a]
$LSD_{0.05}$	0.6	3.2	3.2

Purity = 100 x total PLs as quantified by HPLC / quantity of PL fraction
Same letter in the same column indicates that is not significant difference between the two scales of processing.
$LSD_{0.05}$: Least significant difference at P=0.05.
n, number of replicates in each scale

Table 3. PL content and distribution in the main fractions

Scale of fractionation		NL-1	NL-2	PLs
PL content, %	Analytical (n=2)	1.8±0.4[a]	7.9±0.3[a]	95.9±0.3[a]
	Large (n=3)	1.7±0.6[a]	4.3±0.8[b]	94.9±0.1[b]
$LSD_{0.05}$		1.5	1.9	0.5
PL distribution, %	Analytical (n=2)	2.9±0.7[a]	1.3±0.1[a]	95.9±0.6[a]
	Large (n=3)	3.1±1.1[a]	0.5±0.1[b]	96.4±1.2[a]
$LSD_{0.05}$		2.9	0.2	3.0

Same letter in the same column indicates that is not significant difference between the two scales of processing.

$LSD_{0.05}$: Least significant difference at P=0.05.

n, number of replicates in each scale.

Table 4. Fatty acid composition (%) of the main PL classes of egg-yolk and soybean lecithin

Source	PL	16:0	18:0	18:1	18:2	18:3	20:4
Soybean	PE	16.0	8.3	6.8	57.3	11.7	--
	PI	22.2	19.3	6.1	43.4	9.3	--
	PC	11.2	11.9	8.6	58.6	9.9	--
Egg yolk	PE	25.0	26.8	20.7	16.3	--	11.2
	PC	35.0	13.4	30.4	18.0	--	3.2

Soybean data from Hammond et al. (in press).

Table 5. Cholesterol distribution among the three major products from each fractionation scale

Fractionation		NL-1	NL-2	PLs
Analytical Scale (n=2)	Cholesterol quantity, mg from 15.3 g dry yolk	52.1±1.4	77.2±27.8	18.6±0.2
	Concentration in Product, %	0.9±0.0 [a]	13.0±4.3 [a]	0.5±0.0 [a]
Large Scale (n=3)	Cholesterol quantity, mg from 91.8 g dry yolk	347.8±28.3	286.8±27.8	379.9±31.7
	Concentration in Product, %	0.9±0.1 [a]	10.8±1.0 [a]	1.9±0.1 [b]
$LSD_{0.05}$		0.2	13.4	0.3

Same abbreviations as in Table 1.
Same letter in the same column indicates that is not significant difference between the two scales of processing.
$LSD_{0.05}$: Least significant difference at P=0.05.
n, number of replicates in each scale

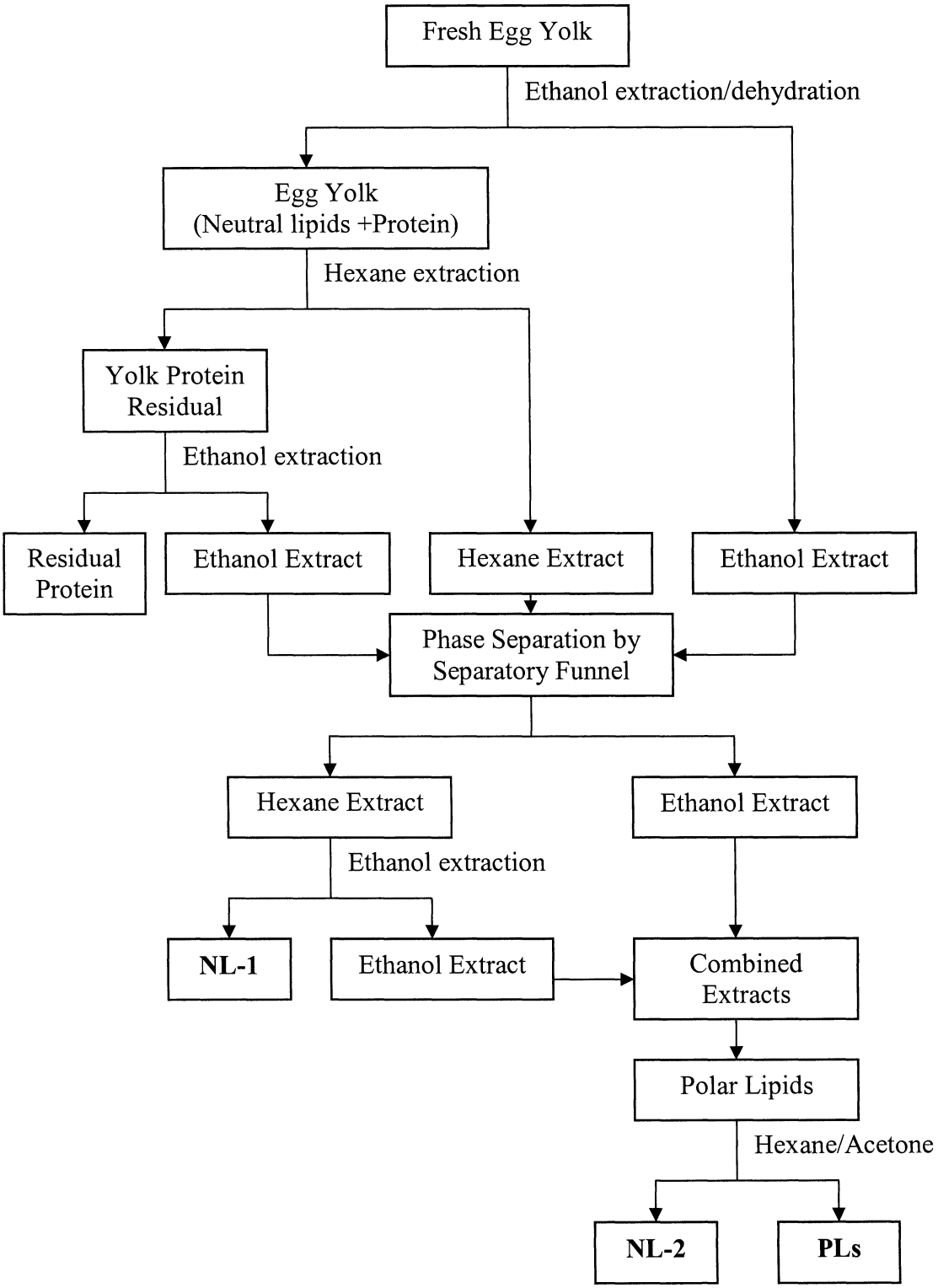

Figure 1. Flow chart of lecithin extraction from fresh egg yolk

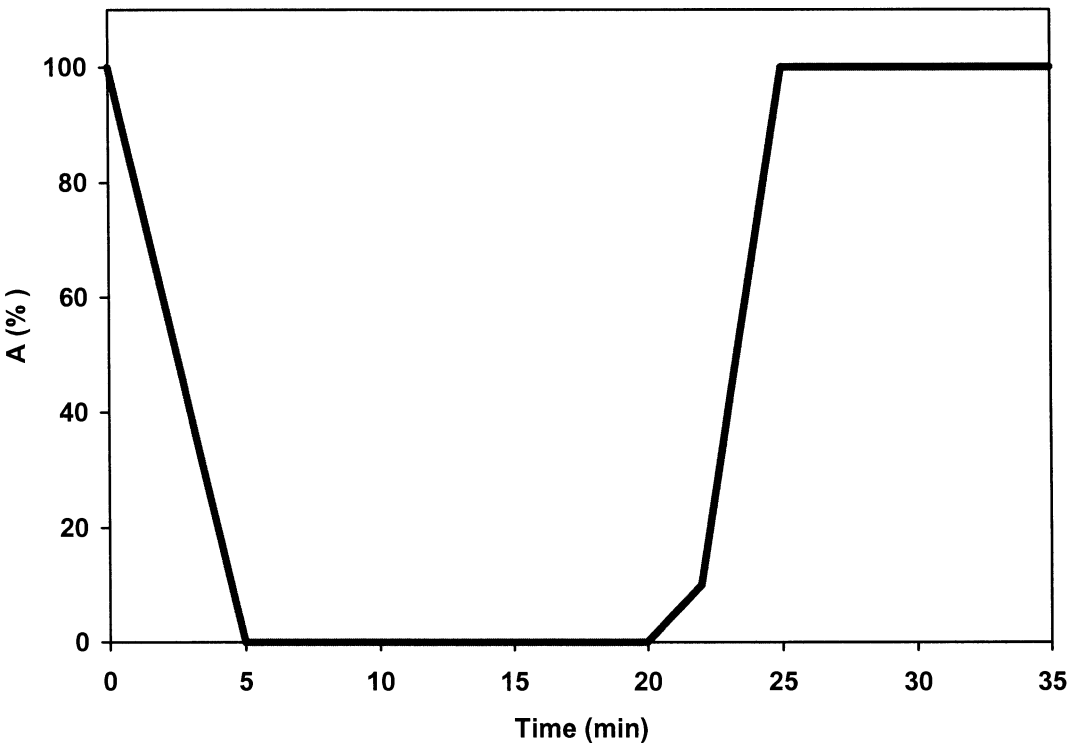

Figure 2. Gradient program of mobile phase for HPLC analysis of PLs.

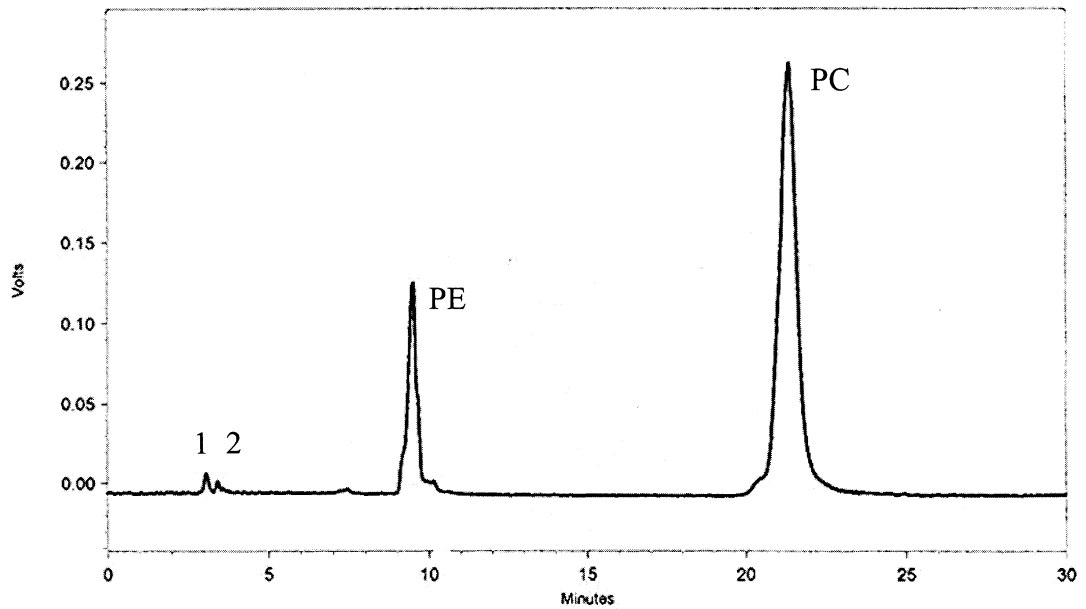

Figure 3. HPLC chromatogram of an egg-yolk PL sample. Peak 1, and 2 were identified as neutral oil and cholesterol, respectively.

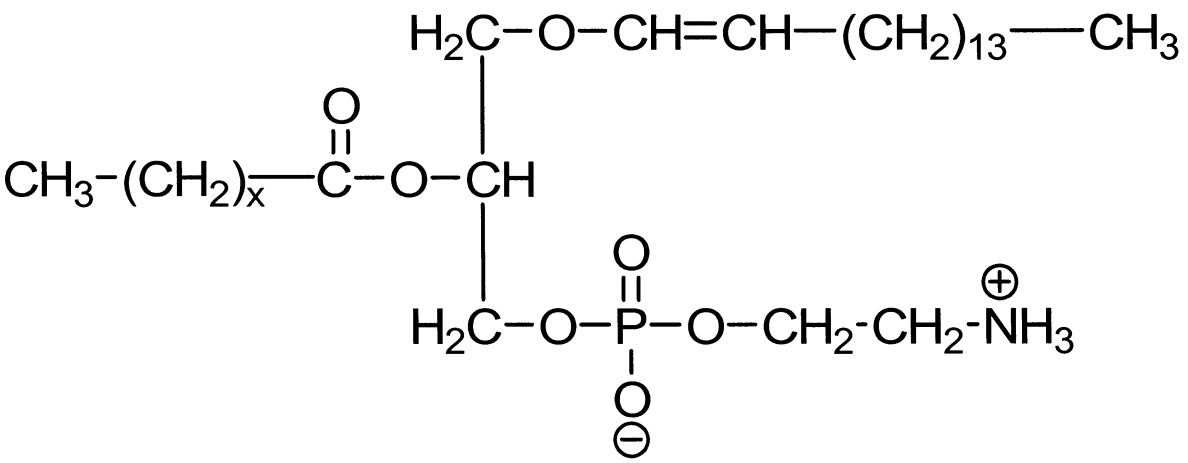

Figure 4. PE plasmalogen identified in egg-yolk lecithin

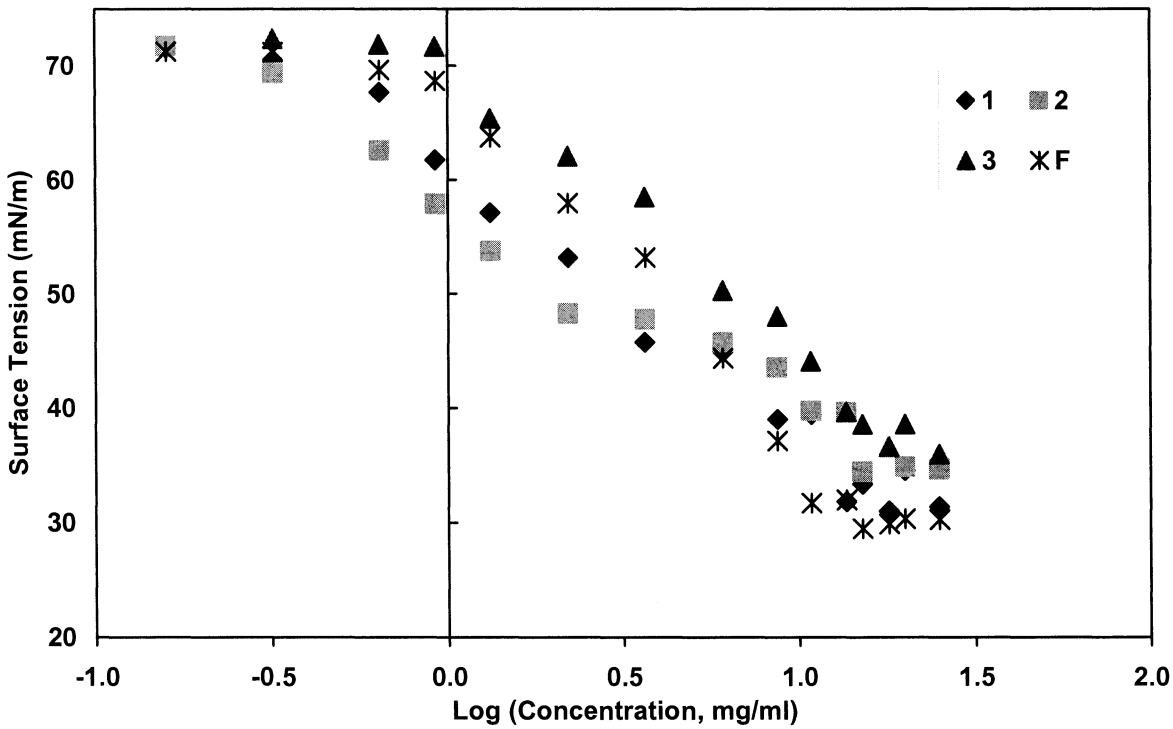

Figure 5. Surface tension reduction of egg-yolk and soy lecithins.
The curves labeled 1, 2, 3, and F are three egg-yolk lecithin products from three replicates of fractionation and a Fisher brand soybean lecithin.

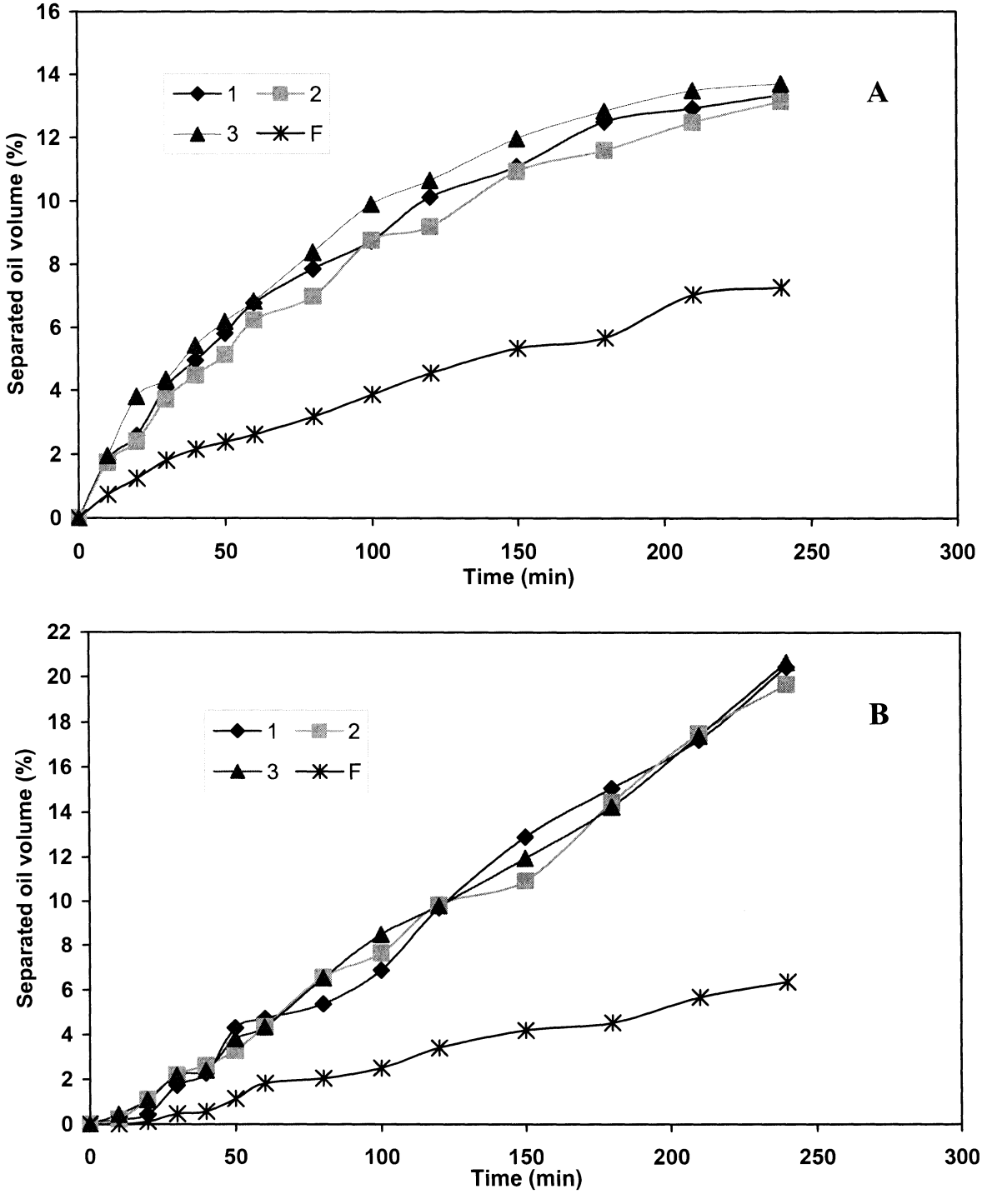

Figure 6. Stability of emulsion (o/w) with 5% (A) and 10% lecithin (B) relative to oil. The curves labeled 1, 2, 3, and F are three egg-yolk lecithin products from three replicates of fractionation and a Fisher brand soybean lecithin.

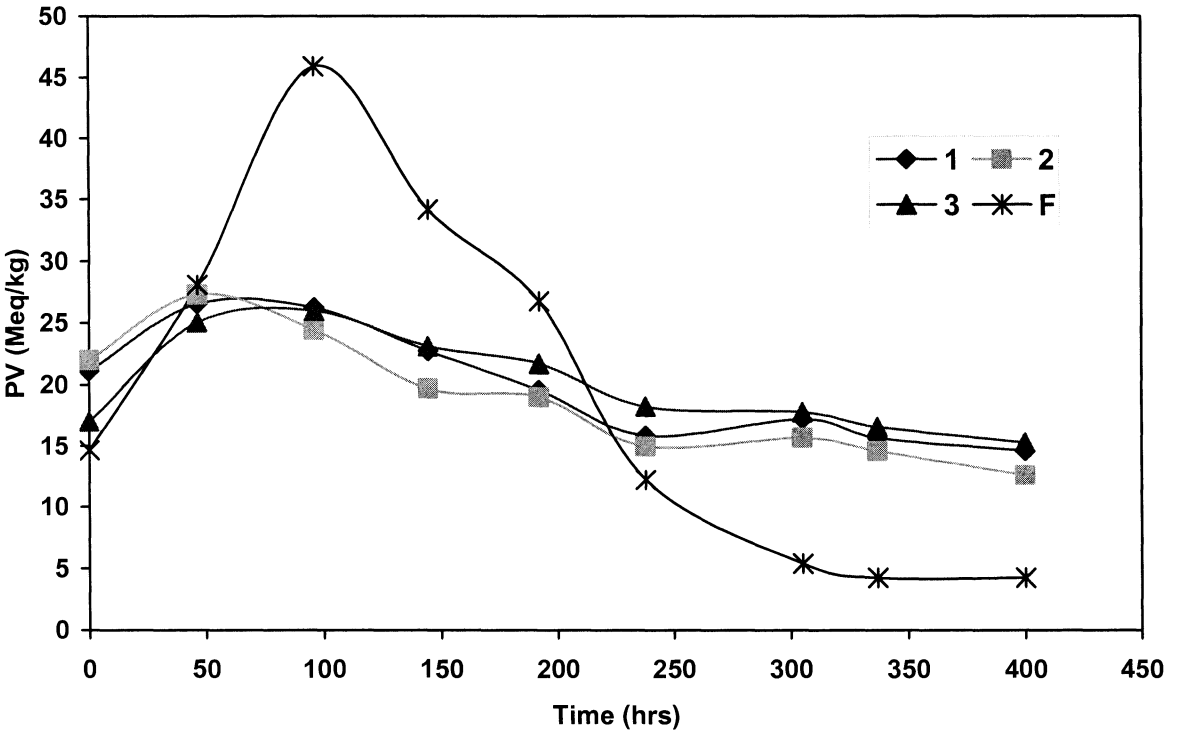

Figure 7. Peroxide values of lecithins quantified by a modified procedure of the ferric thiocyanate method.
The curves labeled 1, 2, 3, and F are three egg-yolk lecithin products from three replicates of fractionation and a Fisher brand soybean lecithin.

CHAPTER 4. GENERAL CONCLUSIONS

The feasibility of direct extraction of phospholipids (PLs) from fresh egg yolk without prior deoiling and the effect of yolk protein denaturation on PL fractionation with aqueous ethanol were studied. Fractionation was performed with deoiled and undeoiled yolk, and with heated and unheated yolks. PL purity and class composition were determined by HPLC with an evaporative light scattering detector. The results showed that the extraction of PLs from fresh egg yolk with ethanol was feasible. Although oil contained in the yolk was partially extracted with ethanol, which decreased the PL purity of the PC-enriched fraction, ethanol extraction facilitated PC enrichment. Egg-yolk protein denaturation was not necessary for the initial PL extraction with ethanol. However, it made the subsequent extraction of the remaining PL fraction from the yolk slightly more efficient. The cholesterol content of the PC-enriched fraction obtained from the undeoiled yolk was much higher than that from the deoiled yolk.

In order to increase PL purity of the fractionated product, a more efficient multiple solvent extraction and partition method was developed. Ethanol and hexane were used to extract the total lipids from the fresh egg yolk. Then, neutral and polar fractions were obtained by phase separation. Finally, acetone was used to precipitate the PLs from the crude polar fraction. Large amount of egg-yolk lecithin was produced using this fractionation method and its functional properties were studied. A surface tension study showed that egg-yolk lecithin and soybean lecithin had similar surface activities. Soybean lecithin created a more stable emulsion than did egg-yolk lecithin. Egg-yolk lecithins oxidized to a maximal

PV of 24 to 27 meq/kg, and soybean lecithin was oxidized to 46 meq/kg before the PV declined, indicating that egg-yolk lecithin is more oxidatively stable than soybean lecithin.

Future research is needed to examine the effect of egg-yolk lecithin on cholesterol absorption in animal models and humans. If the hypothesis of reduction in cholesterol absorption because of egg-yolk lecithin consumption is proved, we indeed will have a valuable multifunctional egg-yolk lecithin product. Future research will also include the study of functional properties of the egg yolk neutral oil and yolk protein and identification of their potential applications in food, feed, and industrial areas.

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