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# Sequential Separation of Main Components from Chicken Egg Yolk

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**Abstract** A study was conducted to develop optimal conditions for a large-scale, sequential separation method for value-added components from egg yolk. Starting with liquid egg yolk, immunoglobulin Y (IgY), phospholipids, and neutral lipids were extracted sequentially using water, ethanol, and n-hexane. The remainder was yolk proteins. Adjusting the pH of diluted egg yolk to pH 5.0-5.2 decreased phospholipids content in the supernatant after centrifugation, which was very important for preventing clogging problem of ultrafiltration filters during the subsequent concentration step for IgY separation. Extraction of precipitants after centrifugation with four volumes of 100% ethanol once removed most of the phospholipids and the purity of phospholipids was more than 85% (wt.) after drying. The subsequent extraction of precipitant from ethanol extraction with four volumes of hexane 3 times removed neutral lipids almost completely and resulted in a high protein product with minimal lipids. The sequential separation method is considered to be advantageous for large-scale separations of many valuable components from egg yolk.

**Keywords:** egg yolk, sequential separation, IgY, lipids, yolk protein

## Introduction

Egg is considered to be a chemical and nutritional store for a potential new life (1). The stagnation of egg consumption for food over the past decades evoked to focus on the use of numerous physiologically active components in egg (2). Although some of the separated egg yolk components are already commercialized, the separation efforts are largely limited to a single component. The main problem is that most of the residuals after isolation of a certain component is discarded, resulting in inefficient and less economical process for separation and utilization of egg yolk components. Egg yolk contains about 51% water, 31% of lipids, 17% of protein, and 1% of ash (3). Yolk lipid can be further divided into three main parts, which are neutral lipids (65.5%), phospholipids (PLs, 28.3%), and cholesterol (5.2%) (4).

Neutral lipid is the major component of egg yolk lipids and can be used as cooking oil or in animal feed. The nutritional value of egg oil is as good as or better than that of plant oils. However, no attempt has been made to separate neutral lipids from egg yolk for commercial use. Phospholipids are excellent emulsifiers and have broad applications for food and non-food products (5).

Phospholipids currently used for foods and non-foods are separated mainly from plant seeds such as soybean, cottonseed, and sunflower. However, the content of phospholipids in soybean lipids is approximately 1/10 of egg yolk lipids. Therefore, egg yolk is much better source for phospholipids than oil seeds. The PLs in egg yolk is composed of 77% phosphatidylcholine (PC), 18% phosphatidylethanolamine (PE) and 3% sphingomyelin (SM) (4). PC has an excellent emulsifying ability that can be used in food processing and contains choline, an important nutrient for human being, especially for infants whose nerve-system is developing quickly. Choline intake is very conducive to brain development and is an important nutrient for liver function and cancer prevention (6). Since increase of cholesterol concentration in blood linked to the risk of arteriosclerosis and coronary diseases, many attempts have been made to remove cholesterol from the lipids of egg yolk. Various membranes were compared to separate cholesterol from the ethanol extracts of egg yolk (7). Jung *et al.* (8) removed over 85% cholesterol in egg yolk using cross linked β-cyclodextrin which were recycled eight times.

The components of egg yolk are distributed in the granules and plasma portions of yolk in a peculiar way. Neutral lipids of yolk granules are present inside the myelin figure and those of plasma are present inside the low density lipoprotein (LDL) spheres surrounded by proteins and phospholipids of LDL (9). Thus, it is impossible to extract neutral lipids from egg yolk without breaking the outer structure of granules and LDL spheres using solvents that can solublize phospholipids, first. Currently, isolation and purification of phospholipids from egg yolk in industrial scale is gradually increasing. However, high amounts of neutral lipid in the remaining residues of egg yolk after phospholipids extraction discouraged the application of the residue for human foods. Neutral lipids can be easily removed from the residue and leaves protein as the major remaining main component of yolk. The proteins recovered after the removal of all the lipids from egg yolk would contain over 90% proteins with well-balanced essential amino acids and can be used as a protein supplement for many food products such as restructured meat, sausage formulation, and others. Jiang and Mine (10) prepared functional oligophosphopeptides from egg yolk phosvitin. Phosvitin separated from egg yolk increased calcium solubility at an ileum condition (11). However, little information is available on the utilization of egg yolk proteins in foods.

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Egg yolk is an excellent source for immunoglobulin since each egg contains 150-200 mg of immunoglobulin Y (IgY). Use of egg yolk antibodies is advantageous over conventional sources (12). The high immunoglobulin content (approximately 10 mg/mL in egg yolks) (13), ease of collection and easy production make IgY a potential source for immunological supplementation of foods. Many researchers (14, 15) have also developed chicken eggs with specific antibodies from immunized hens. Tini *et al.* (16) succeeded in generating antibodies against a-subunit of hypoxia inducible factor-1 (HIF-1) in chicken and its application. Purified antibodies can be used in various areas such as cosmetics to cure skin diseases caused by certain microorganisms, in an infant formula to prevent certain disease or to spray on the surface of carcass to prevent the growth of microorganisms causing disease. Shimizu *et al.* (17) reported that anti-*E. coli* IgY aggregated the bacterial cells and inhibited their growth *in vitro*. Oral administration of purified antibodies had been successfully used to prevent specific diseases in infants, fish, and small animals.

There had been sporadic attempts to separate few valuable components from egg yolk individually, but no systemic approach was found to separate multiple components from egg yolk sequentially. Most of the separating operations focused on single component isolation neglecting further use of the remainder. The objectives of this research were to compare currently available preparation methods for value-added components from liquid egg yolk and to develop optimal continuous processes for the separation of IgY, neutral lipids, phospholipids, and yolk proteins from water-soluble and water-insoluble yolk components. Developing sequential separation methods using water, ethanol, and n-hexane will bring high efficiency in separating many important components from egg yolk and increase the value and utilization of egg yolk.

## Materials and Methods

**Water extraction of egg yolk and IgY separation** Eggs obtained from a local market were broken and the yolk was separated from the egg white. Egg yolk was diluted ten times (w/v) with distilled water, and water-soluble and water-insoluble components were separated using centrifugation. Combinations of different pHs (6.3 and 5.0, adjusted with 1 N HCl), 6 sample holding times (0, 1, 2, 4, 8, and 24 hr at 4°C), and 2 centrifugation times (30 min and 60 min at 2,800×g) were tested to find the optimal conditions for the quality and quantity of water-soluble components from egg yolk. The water-soluble supernatant that contains IgY was separated from water-insoluble part using the best sample preparation conditions selected and used for the rest of the sequential isolation.

Solid contents of supernatants were measured after drying the extract completely at 105°C. Protein concentration in the supernatant was determined using the Bradford method (18). While adding the Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA), the test tube was vortex mixed gently to make sure thorough mixing. After incubating samples for 15 min absorbance was measured at 595 nm. Protein content (N×6.25) in the solids of supernatants was determined according to the Micro-Kjeldahl method (19).

To concentrate IgY in the supernatant, ultrafiltration was applied using a millipore hollow fiber cartridge (Amicon Inc., Beverly, MA, USA) with a molecular weight cut-off size of 30 kDa. Before ultrafiltration, the sample was filtered through a Whatman No. 1 filter paper (Kent, UK) to remove fine water-insoluble particles, which may obstruct the pores in the cartridge. The ultrafiltration process was done at a constant inlet pressure of 25 psi. The sample was run until it was 10 times concentrated. After concentrating, the sample was filtered through a Whatman No. 42 filter paper (Kent, UK) to remove particles. For concentrating small samples amicon centricones (Amicon) with cut-off size of 10 kDa were used.

**Determination of the purity of separated IgY by SDS-PAGE** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under the non-reducing conditions was carried out to check the status of IgY using a Mini-Protein® 3 Cell (Bio-Rad Lab., Hercules, CA, USA). A 7 or 8% of running gel and a 4% stacking gel was prepared depending on the requirement. The protein was stained with a Coomassie brilliant blue R-250 (Sigma-Aldrich) in 10% acetic acid and 30% methanol. The molecular size markers used were ranged from 10 kDa to 250 kDa (Bio-Rad Lab., Hercules, CA, USA) to evaluate the IgY in the water extract.

**Determination of activity of the separated IgY by ELISA** Immulon I micro titer 96-well plates were used as the solid support (17). Polystyrene 96-well plates (Nunc Inc., Rochester, NY, USA) were coated with 100 μL of IgY samples dissolved in coating solution (0.05M carbonate buffer, pH 9.6) and incubated over night at 4°C. After washing the wells three times with phosphate buffered saline (PBS-TW: 10 mM phosphate, 0.15 M NaCl, pH 7.2, 0.05% Tween 20), 300 μL/well of blocking solution (1% bovine serum albumin solution in PBS was added. After incubating for 1 hr at room temperature, the plate was washed with PBS-TW. To each well of the plate, 100 μL primary antichicken IgG (1: 10,000 solution diluted with 1% BSA)-conjugated alkaline phosphatase was added and then incubated for 1 hr. After washing with PBS-TW, each well was added with 50 μL of *p*-nitrophenyl phosphate solution as a substrate for color development and incubated for 30 min. The enzyme reaction was stopped by adding 50 μL of 3 N NaOH and the color developed was read on an enzyme-linked immunoso2bent assay (ELISA) plate reader (Molecular Devices, Canton, USA) with a 405 nm filter. For each plate, three controls were prepared: one positive control with reagent- grade chicken IgG (Sigma-Aldrich), a non-specific antigen BSA as another control, and a negative control without antigen. All the procedures for ELISA were conducted at room temperature (about 25°C).

**Separation of phospholipids and neutral lipids from water insoluble fraction of egg yolk** For the extraction of phospholipids and neutral lipids from the water-insoluble fraction of egg yolk, three food-grade solvents (ethanol, acetone, and hexane) were tested. Solvent was

added to the water-insoluble fraction of yolk, homogenized using a hand-held blender (KitchenAid, St. Joseph, MI, USA) for 2 min, and then centrifuged at 2,000×g for 10 min. The supernatant was collected and the solvent was removed using a rotary evaporator under vacuum. The purity of phospholipids and neutral lipids from each solvent extraction and after further purification was determined using a thin-layer chromatography.

**Determination of lipid classes with thin layer chromatography** Dried solvent extracts were dissolved in Folch solution (chloroform: methanol = 2:1) (20) to make the lipid content approximately 100 mg/mL. Two drops of absolute ethanol were added to the sample to help solublizing phospholipids. Sample and standards (100 μL each) were applied onto pre-coated silica gel-G plate (20× 20 cm, Merck, Whitehouse itation, NJ, USA) that had been previously activated by heating at 120°C for 2 hr. The plates were developed in chloroform: methanol: water (98:38:6) solution to 10 cm from the origin (about 40 min). The plate was air dried and then developed in hexane and diethyl ether (120: 30) solution for 20 min. The plates were air-dried and then sprayed with 0.1% (w/v) 2'7'-dichlorofluororescein in ethanol. The spots corresponding to PE, PC, cholesterol, and triglyceride (TG) were identified under UV light.

## Results and Discussion

Consecutive isolation of IgY, phospholipids, and neutral lipids from whole egg yolk was done successfully using pH 5.0 water, four volumes of anhydrous alcohol and n-hexane for food usage. IgY and phospholipids are most widely isolated from egg yolk for food and medicinal usage worldwide. However, the remainder of each isolated component is discarded. With increasing interest in physiological functions of various components in egg yolk sequential separation of components from egg yolk without serious loss of intact characteristics of each molecule is needed with respect to process efficiency.

**Variables on IgY extraction** pH adjustment of diluted egg yolk to 5.0 increased the amounts of proteins and immunoglobulins extracted but the profile of proteins extracted was not affected (Fig. 1 and 2). Lipid content and the turbidity of the supernatant decreased dramatically by lowering pH of the 10-fold diluted egg yolk, which is very important for the subsequent ultrafiltration step to concentrate the supernatant (Table 1). Holding of diluted egg yolk in cold room up to 24 hr and the duration of centrifugation (30 vs. 60 min at 2,800×g) had practi-cally no effect on protein and IgY extraction (Fig. 1 and 2). As both the holding time and centrifugation time are expensive and rate limiting factors for a continuous, large-scale separation of IgY from egg yolk, holding time before centrifugation is not necessary and centrifugation time should be limited to 30 min at 2,800×*g*.

Akita and Nakai (21) compared water dilution method with polyethyleneglycol, dextran sulfate, and xanthan gum precipitation methods and found that water dilution method was superior to the other three precipitation methods in terms of IgY yield, purity, ease of use,

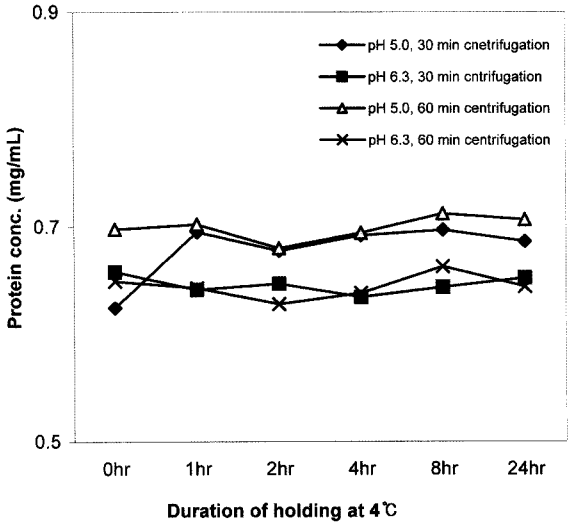


**Fig. 1. Effects of pH of egg yolk diluents, holding time before centrifugation, and the duration of centrifugation at 2,800×g on protein concentrations of supernatant.**

potential scaling up, and immunoactivity of IgY. Increasing dilution ratio up to 10 times facilitated the precipitation of lipoproteins in egg yolk because lower ionic strength helped the aggregation of lipoproteins in egg yolk. However, volume increase makes it difficult to handle for the subsequent preparation steps of IgY purification. Bade and Stegeman (22) extracted immunoglobulin directly from egg yolk by precipitating lipids with cold propane-2-ol and Devi *et al.* (23) used two times dilution of yolk with distilled water and then treated them with a -70°C freeze-thaw cycling. These methods eliminated concentration step for immunoglobulin extract, but the yield of immuno-globulin was lower than the higher dilution method.

To reduce volume and concentrate supernatant, ultrafiltration and precipitation of proteins with ammonium sulfate were suggested (24-27). Between the two methods, however, ultrafiltration was more practical than ammonium sulfate precipitation for scaling up, continuous processing for IgY purification, and further separation of other components. In using ultrafiltration, a pre-treatment that removes lipids and lipoproteins is essential because they clog filters and reduce the productivity of ultrafiltration process. Various methods such as adding polyethylene glycol (12, 28), dextran sulfate (29), γ-carrageenan gum, xanthan gum (30, 31), hydroxypropylmethyl cellulose phthalate (32), caprylic acid (33) or pectin (15), adjusting pH (34), or freeze-thaw cycling had been suggested to remove lipids and lipoproteins from water-diluted egg yolk or egg yolk extract. Among these methods, pH adjustment was the most practical for scaling up and continuous

**Table 1. Effect of pH adjustment on lipid content and turbidity of egg yolk supernatant after centrifugation**

| Treatment | Lipid (%) | Turbidity[1)] |
|---|---|---|
| Control (pH 6.3) | 1.00 | 2.97 |
| pH adjustment to 5.0 | 0.08 | 0.33 |

[1)]Turbidity is the absorbance of sample at 600 nm. n = 4.

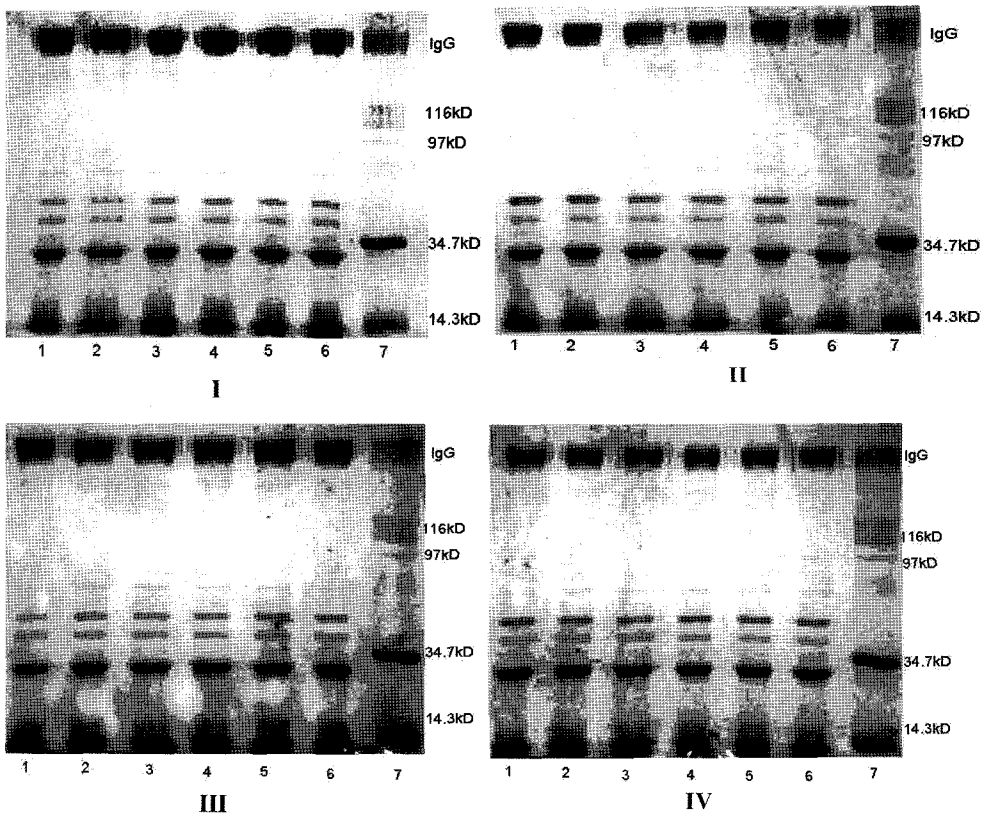


**Fig. 2. SDS-PAGE for various water-soluble fraction of egg yolk sample.** I & III - at pH 5.0 and II & IV - at pH 6.3. I & II - centrifuged for 30 min and III & IV - centrifuged for 60 min at 2,800×g. Lane 1 - samples incubated for 0 hr, lane 2 - 1 hr, lane 3 - 2 hr, lane 4 - 4 hr, lane 5 - 8 hr, lane 6 - 24 hr and lane 7 - molecular size markers.

processing, and the yield of IgY was the highest. As shown in Table 1, adjusting pH to 5.0 eliminated the lipids from the supernatant almost completely. However, lowering pH below 5.0 is not recommended because at pH<5.0, the precipitants became loose and watery due to over-hydration of proteins. The pH adjustment of diluted egg yolk to pH 5.0 significantly increased moisture content in the precipitant (water-insoluble fraction of yolk) after centrifugation. This happened because the adjusted pH 5.0 is lower than the isoelectric pHs of all egg yolk proteins and water molecules tend to bind proteins when the pH is lower or higher than the isoelectric pHs of a protein. About 15% of IgY was lost at ultrafiltration step (Table 2). Significant amount of concentrated IgY was lost in membrane of filter, tubing and empty spaces of filter. This problem, however, can be minimized in large-scale or continuous processing of ultrafiltration, instead of batch-type processing.

Since yolk contains almost all the antibody in egg, egg yolk can be mixed in animal feed directly as an antibody source. Direct use of egg yolk as an antibody source is simple and costs less, but its application and efficiency is limited because the concentration of antibody is low. Additional process such as defatting step can be applied to concentrate antibodies from egg yolk (35). Supercritical fluid extraction and organic solvent extraction methods are currently used as defatting methods for egg yolk (36). Most of the current utilization of immunoglobulin (IgY) from egg yolk is a direct use of egg yolk obtained from

**Table 2. Amount of IgY[1)] (mg) recovered at each separation step[2)]**

| | IgY (mg) | % of total |
|---|---|---|
| Total IgY | 883.0 | 100 |
| After water extraction (10 ×) | 845.0 | 95.9 |
| After ultrafiltration | 677.5 | 80.0 |

[1)]On the basis of IgY activity determined by ELISA. Values are average of 2 replications.
[2)]Based on 100 g of liquid egg yolk as a starting material.

hens immunized for specific diseases. Spray-drying of the supernatant will produce a water-soluble egg yolk product with high IgY, which can be used as a dietary supplement. However, if antibodies from egg yolk are isolated and purified, their efficiency and application can be expanded significantly.

**Extraction of neutral lipid and phospholipids from water-insoluble fraction of egg yolk** Because neutral lipids and phospholipids separated from the water-insoluble fraction of diluted egg yolk are aimed for human consumption, solvents of choices are limited to ethanol, hexane, and acetone. Both ethanol and acetone had strong extracting capabilities for phospholipids, but hexane was able to extract very small amount of lipids from the water-insoluble fraction of egg yolk (Fig. 3). The major component of both ethanol and acetone extract was phospholipids, but some neutral lipids and cholesterol were also extracted as

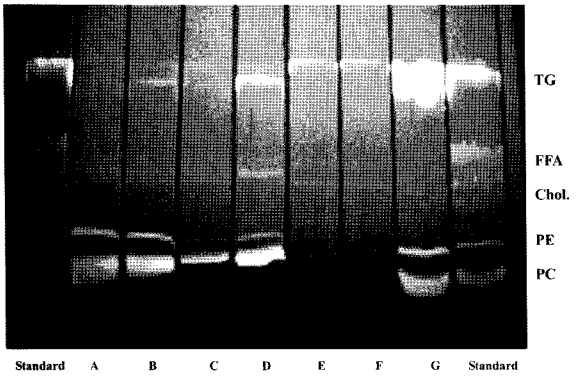


**Fig. 3. Thin layer chromatogram for component distribution of various egg yolk extracts.** A: extraction with 9 vol. of ethanol, B: extraction with 4 vol. of ethanol, C: extraction with 9 vol. of acetone, D: extraction with 4 vol. of acetone, E: extraction with 4 vol. of hexane, F: extraction with 9 vol. of hexane, G: extraction with 4 vol. of hexane after 4 volume ethanol extraction. TG: triglycerides; FFA: free fatty acid; chol: cholesterol; PE: phosphatidylethanolamine; PC: phosphatidylcholine.

the volume of those solvents increased, especially with acetone. Using 4 volumes of ethanol or acetone was better than 9 volumes of ethanol or acetone because very high-purity (>85%) phospholipids with little neutral lipids could be obtained. Repeated extraction of the precipitant of ethanol extraction with 4 volumes of ethanol increased the amounts of neutral lipids and cholesterol in the extract. It is also clear that decrease in ethanol concentration less than 4 volumes decreased its extractability.

Both ethanol and acetone are approved for extracting lipids for human consumption in some countries. However, using 4 volumes of ethanol was better than that of acetone because ethanol had higher extractability for phospholipids from the water-insoluble fraction of egg yolk than acetone (Fig. 3). It is very important point because almost all neutral lipids are eliminated from the ethanol extract without further purification steps. The remaining lipid (mainly neutral lipids) after ethanol extraction was extracted using hexane. Hexane is the most widely used solvent in extracting lipids from plant seeds and also has a strong extracting power for neutral lipids. Thus, hexane was selected as a second solvent to extract neutral lipids from the solids of ethanol extraction. As expected, the second (hexane) extract was mainly composed of neutral lipids but contained some phospholipids (PC and PE), indicating that there are still significant amount of phospholipids left in the solids after the first ethanol extraction (Fig. 3). Four volumes of hexane were used to remove remaining lipids from the solids of ethanol extraction and the extraction was repeated 3 times. Earlier, Sim (37) used repeated ethanol extraction at different solvent temperatures followed by cold temperature crystallization and filtration to extract and separate neutral lipids and phospholipids from liquid egg yolk. Chromatography also has been proposed to further purify PLs by using alumina, silica gel, and ion exchange cellulose as media (7). The precipitants obtained after the second solvent extraction with hexane contained mainly proteins. After drying, the final egg yolk residue contained more than 90% of proteins with small amounts of lipids and carbohydrates (Table 3).

**Overall scheme for sequential separation** Sequential separation of IgY, phospholipids, neutral lipids, and yolk protein was successfully undertaken starting from egg yolk. Dilution of egg yolk with 9 volumes of water adjusted at pH 5 was selected as the first step for the consecutive separation of the components. The diluted egg yolk was centrifuged at 2800×g and 4°C for 30 min. Ultrafiltration of the supernatant with a molecular weight cut-off size of 30 kDa concentrated IgY. The residues of centrifugation, water insoluble fraction of the diluted egg yolk, was treated with 4 volumes of 100% ethanol and centrifuged at 2800×g for 10 min. Evaporation of the ethanol under vacuum recovered phospholipids. Further extraction of the precipitant with 4 volumes of hexane three times and centrifugation at 2800×g for 5 min isolated proteins. Hexane was removed from the super-natant to obtain neutral lipids. Removal of phospholipids using 2 volumes of 100% ethanol improved the purity of the neutral lipids.

Overall mass balance and component mass balance during consecutive extraction of egg yolk with water,

**Table 3. Amount (g) of the yolk solids distributed to the supernatants and precipitants at each extraction step[1)]**

| | Protein | Phospholipids | Cholesterol | Neutral lipids | Ash |
|---|---|---|---|---|---|
| *Supernatant* | | | | | |
| Water extraction | 3.5±0.21[2)] | 0.2±0.03 | - | 0.3±0.02 | - |
| Ethanol extraction | 0.4±0.07 | 9.4±0.17 | 1.2±0.24 | 0.3±0.04 | - |
| Hexane extraction | - | 1.2±0.19 | 0.1±0.04 | 20.2±0.33 | - |
| *Precipitant* | | | | | |
| Water extraction | 13.6±0.32 | 10.6±0.38 | 1.4±0.32 | 20.6±0.44 | 0.7±0.36 |
| Ethanol extraction | 13.2±0.16 | 1.2±0.11 | 0.2±0.04 | 20.3±0.18 | 0.7±0.19 |
| Hexane extraction | 13.2±0.19 | - | - | 0.1±0.03 | 0.7±0.17 |

[1)]Based on 100g of liquid egg yolk as a starting material. n = 4.
[2)]Average±standard deviation.

**Table 4. Lipid composition (wt%) of solvent extracts[1)]**

| | Ethanol extract | Hexane extract following ethanol extraction |
|---|---|---|
| Phospholipids | 86.2±0.16 | 5.6±0.16 |
| Cholesterol | 11.0±0.24 | - |
| Neutral lipid | 2.8±0.43 | 94.0±0.29 |

[1)]Average ± standard deviation. n = 4.

ethanol, and hexane are shown in Table 3. Thirty seven grams out of 51 g of total egg yolk solids from 100 g egg yolk remained in the precipitant fraction and 4 g (3.5 g proteins, 0.5 g lipids) went to the water-soluble fraction after water extraction. Considering 100 g egg yolk contain 17 g of proteins (5), approximately 20% of the whole egg yolk proteins went to water-soluble fraction. Thus, a plan to recycle water-soluble proteins from the supernatant after recovering IgY may be needed in the future. Ethanol extract contained 11.5 g of solids most of which was phospholipids (9.4 g) and some cholesterol (1.1 g). The amounts of proteins and neutral lipids were small, 0.4 and 0.3 g, respectively. Table 4 revealed that phospholipids content of the ethanol extract was more than 85%. Therefore, phospholipids obtained from single ethanol extraction can be used in food products without further purification. However, due to somewhat high cholesterol content in the ethanol extract, further purification to remove cholesterol may be necessary. The subsequent hexane extraction of the precipitant from ethanol extraction removed 21.5 g of lipids, which was composed of 20.2 g neutral lipids and 1.2 g phospholipids (Table 3). Phospholipids in hexane extract can be removed by repeated extraction with ethanol. The degree of purity should be decided by the specific applications or needs for phospholipids and neutral lipids. The final precipitant after hexane extraction was 14 g and was composed of 96% proteins, 3% ash and less than 1% of lipids on a dry weight basis. Moisture content of the final precipitant after hexane extraction was less than 7%. This product can be a useful source for protein supplementation of other food products or can be used as a starting material for other valuable components, such as phosvitin.

The process is significant because many valuable components in egg yolk can be sequentially separated and used for various food and non-food purposes. With this sequential separation process of egg yolk components, natural resources can be maximally utilized with minimal wastes. Costs for separating value-added components from egg yolk can be decreased.

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