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Distribution of Phospholipids, Cholesterol and Carotenoids in Two-Solvent System during Egg Yolk Oil Solvent Extraction

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Abstract**—**Egg yolk oil is a concentrated source of egg bioactive compounds, such as fat-soluble vitamins, phospholipids, cholesterol, carotenoids and others. To extract lipids and other fat-soluble nutrients from liquid egg yolk, a two-step extraction process involving polar (ethanol) and non-polar (hexane) solvents were used. This extraction technique was based on egg yolk bioactive compounds polarities, where non-polar compound was extracted into non-polar hexane, but polar in to polar alcohol/water phase. But many egg yolk bioactive compounds are not strongly polar or non-polar. Egg yolk phospholipids, cholesterol and pigments are amphipatic (have both polar and non-polar regions) and their behavior in ethanol/hexane solvent system is not clear. The aim of this study was to clarify the behavior of phospholipids, cholesterol and carotenoids during extraction of egg yolk oil with ethanol and hexane and determine the loss of these compounds in egg yolk oil. Egg yolks and egg yolk oil were analyzed for phospholipids (phosphatidylcholine (PC) and phosphatidylethanolamine (PE)), cholesterol and carotenoids (lutein, zeaxanthin, canthaxanthin and β-carotene) content using GC-FID and HPLC methods. PC and PE are polar lipids and were extracted into polar ethanol phase. Concentration of PC in ethanol was 97.89% and PE 99.81% from total egg yolk phospholipids. Due to cholesterol's partial extraction into ethanol, cholesterol content in egg yolk oil was reduced in comparison to its total content presented in egg yolk lipids. The highest amount of lutein and zeaxanthin was concentrated in ethanol extract. The opposite situation was observed with canthaxanthin and β-carotene, which became the main pigments of egg yolk oil.

Keywords**—**Cholesterol, egg yolk oil, lutein, phospholipids, solvent extraction.

I. Introduction

THE extraction of egg yolk oil from liquid egg yolk meets several technical problems which are related to a high water content of egg yolk and egg yolk proteins. The main bioactive compounds of egg yolk are non-polar and need to be extracted with non-polar solvents. But application of non-polar solvent directly to liquid egg yolk is senseless.

Egg yolk lipids are associated with proteins, called lipoproteins. The lipoprotein is basically a carrier for cholesterol and other fat-soluble compounds like vitamins A, D, E and carotenoids [1].

To access lipids and other fat-soluble nutrients from lipoproteins, proteins must be denaturized. Usage of two-step extraction process involving polar and non-polar solvents can solve the problem. As a first step, polar alcohol can be used to denaturate proteins and secondly, precipitate can be extracted with non-polar hexane. As a result, high purity egg yolk oil can be obtained [2]. This technique is based on compound polarities, where polar compounds are concentrated in polar alcohol/water phase, but non-polar in hexane phase. But not all egg yolk compounds are strongly polar or non-polar. Many bioactive egg yolk compounds such as phospholipids, cholesterol and pigments have both polar and non-polar regions and their behavior in two-solvent system can be predictable, but not totally clear.

A. Phospholipids

Phospholipid content in egg yolk is approximately 10%. The main egg yolk phospholipids are PC and PE whose content from total phospholipids in egg yolk are 80% and 18%, respectively [3], [4].

Phospholipids are amphipathic molecules, which mean that they have both hydrophilic and hydrophobic regions. One end of the molecule (the fatty acid chains), is highly non-polar because only carbons and hydrogens are present. The other end of the molecule, by contrast, is polar - it contains the highly polar phosphate group and polar molecules such as choline and ethanolamine (Fig. 1).

For extraction of phospholipids from egg yolk, ethanol usually is used [4], what means that phospholipids are polar compounds. Taking into account high solubility of phospholipids in ethanol, there is no doubt that they will be totally extracted in polar ethanol/water phase.

B. Cholesterol

Many health concerns are related to cholesterol. The more prevalent of them is coronary heart disease [5]. But in reasonable levels, cholesterol is good for health, because it is precursor of vitamin D_3, bile acid and some steroidal hormones [6]. Cholesterol presence in egg yolk oil can be rated both as benefit or disadvantage - the matter is its concentration.

The cholesterol, same as phospholipids, in eggs is carried by lipoproteins [1]. The breakage of lipoprotein structure by organic solvents results in the release of free cholesterol and its following extraction in specific solvent will depend from polarity of extraction solvents and cholesterol.

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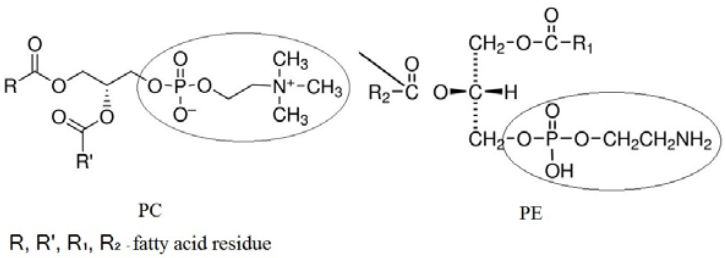

Fig. 1 Structural formulas of PC and PE [7]

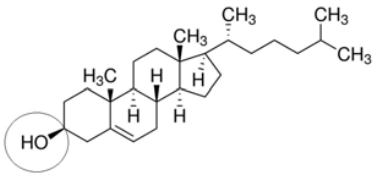

Fig. 2 Structural formula of cholesterol [7]

Cholesterol is another example of an amphipathic molecule. Cholesterol has a hydroxyl group and a steroid ring structure with hydrocarbon tail (Fig. 2). It means that cholesterol molecule contains a small polar region, but entire cholesterol molecule is non-polar.

The main point is the solubility of cholesterol in ethanol/water mixture and hexane. Cholesterol is practically insoluble in water (0.2 mg $100ml^{-1}$) and slightly soluble in ethanol where solubility increases with temperature [8].

Hexane is widely used as a solvent for non-polar lipid extraction. Its main use is to extract neutral lipids from mixtures of water with alcohols [9]. In our research egg yolk oil was extracted using similar solvent mixture where neutral lipids are extracted with non-polar hexane from polar ethanol/water phase when both ethanol and hexane extract are mixed together. As cholesterol molecule has a small polar region we predict that most of it will be extracted in non-polar hexane phase.

C. Carotenoids

Carotenoids are non-polar compounds, which are divided into two subclasses, more polar compounds called xanthophylls, or oxycarotenoids, and the non-polar carotenes [9]. Egg yolk carotenoids are presented by lutein, zeaxanthin, canthaxanthin and β-carotene [11].

Lutein and zeaxanthin belong to xanthophylls and are more polar from all egg yolk carotenoids because both contain hydroxyl groups. Furthermore, xanthophylls may be present both in free form and as fatty acid esters, which may affect their polarity and hence their solubility [12].

In other studies, mixtures of petroleum ether and alcohol were used for the extraction of total carotenoids, separating the xanthophylls into the alcohol phase and the non-polar carotenes into the ether phase [13], [14]. Probably the same will be observed using polar ethanol and non-polar hexane.

Although lutein and zeaxanthin have identical chemical formulas they are not stereoisomers. Lutein and zeaxanthin are polyisoprenoids containing 40 carbon atoms and cyclic structures at each end of their conjugated chains. The main difference between them is in the location of a double bond in one of the end rings giving lutein three chiral centers as opposed to two in zeaxanthin (Fig. 3).

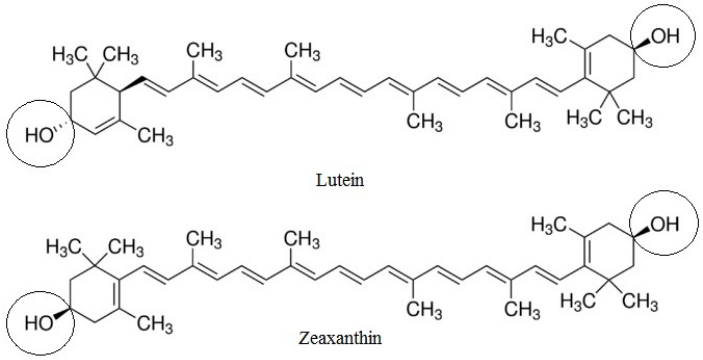

Fig. 3 Structural formulas of lutein and zeaxanthin [7]

Canthaxanthin consists predominantly of trans-ß-carotene-4,4'-dione (Fig. 4) together with some amounts of other isomers. Canthaxanthin is insoluble in water and ethanol, practically insoluble in vegetable oils, very slightly soluble in acetone and methanol [15].

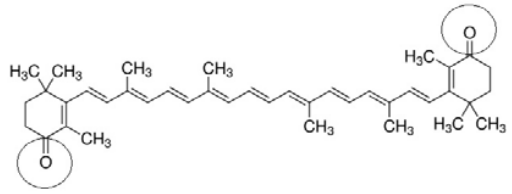

Fig. 4 Structural formula of canthaxanthin [7]

β-carotene is a hydrocarbon without functional groups therefore it is very lipophilic compound (Fig. 5). It can be extracted from egg yolk with a non-polar solvent such as hexane [16].

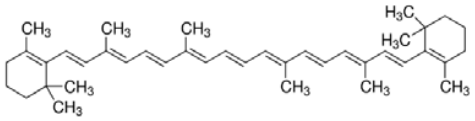

Fig. 5 Structural formula of β-carotene [7]

The separation of β-carotene from the mixture of other carotenoids is based on the polarity of a compound [16]. In our case where solvent mixture contains polar ethanol and non-polar hexane, β-carotene, as a most non-polar from carotenoids, will be extracted in hexane phase also containing neutral lipids. Egg yolk oil obtained by two-stage extraction with ethanol and hexane must contain all β-carotene presented in egg yolk.

Because the behavior of egg yolk bioactive compound in two-solvent system is not totally clear, the aim of this study was to analyze the distribution of phospholipids, cholesterol and carotenoids during egg yolk oil solvent extraction and determine content of these compounds in egg yolk oil (neutral lipids).

II. Materials and Methods

A. Materials

For the egg yolk oil extraction, thirty eggs, purchased in the local store, were cracked and yolks were separated from egg whites. Egg yolks were pooled together and homogenized getting one pooled sample.

Extraction solvents (ethanol and hexane), used in egg yolk oil extraction, were analytical grade from Sigma Aldrich (Germany). Compressed nitrogen gas with purity 99.999% HiQ Nitrogen 5.0 was from Linde AG (Germany).

B. Egg Yolk Oil Extraction

Lipid extraction with ethanol and hexane from liquid egg yolk was made by following steps. First, polar lipids were extracted with ethanol from liquid egg yolk and then neutral lipids were extracted from precipitate with hexane. The extracts were filtered by vacuum filtration and supernatants were collected and added to the same separatory funnel. Both ethanol and hexane extracts were thoroughly but gently, to avoid emulsion formation, mixed to extract polar lipids and impurities to a polar ethanol/water phase and neutral lipids to a non-polar hexane phase. Then the mixed extracts were left for 1 hour for phase separation. Bottom ethanol/water layer, containing polar lipids and water soluble compounds was drained from the separatory funnel through the open stopcock. Hexane extract was collected in clean container. Egg yolk oil was obtained from the hexane extract by evaporation of the hexane in the rotary evaporator IKA RV 10 Control V (IKA®-Werke GmbH and Co. KG) at the temperature of 70°C and 400 mbar pressure. After solvent evaporation in the rotary evaporator, as the last step of the solvent removal, the pure nitrogen gas was laid through the egg oil for 10 minutes in the same rotary evaporator with the same evaporation conditions by means of a plastic tube immersed in the oil [2].

C. Analysis

Phospholipids, cholesterol and carotenoids were determined in liquid egg yolk and egg yolk oil. Phospholipids, cholesterol and carotenoids content in polar (ethanol/water) extraction phase was expressed as a difference between these compound content in egg yolk and egg yolk oil.

1. Egg Yolk Total Lipids

Total lipids of egg yolk were determined using the standard method procedure [17] using chloroform and methanol (2:1).

2. Phospholipids

For separation of phospholipids approximately 5 g of egg yolk lipid sample or egg yolk oil were fractionated on a 5-g column of silica gel (60-200 mesh), by sequential elution with 200 ml chloroform, 100 ml acetone, 100 ml methanol and 100 ml 0.1% phosphoric acid in methanol. The methanol fractions were combined for recovery of the total PL. Solvent was removed in a rotary evaporator. The sample residue was dissolved in chloroform, washed with saturated salt solution, and then sodium bicarbonate was added until neutral. The sample was dried with sodium sulfate and filtered. The solvent was removed by rotary evaporation at room temperature. Samples were diluted with chloroform to give a 1 mg μl^{-1} solution for analysis [18].

The phospholipids standards of PE and PC, chloroform, methanol, ammonium hydroxide and water for the mobile phase were of HPLC grade and obtained from Sigma-Aldrich (Germany). For determination of phospholipids Shimadzu Nexera X2 with evaporating light scattering detector ELSD-LTII (Japan) was used. As the nebulizing gas, N_2 was used at a flow rate of 4 l min^{-1}, and a nebulizing temperature of 40 °C.

A 125 × 4.0 mm Si - 60 column with 5 µm particle diameter (Lichrospher) was used. The elution program was a linear gradient with 80: 19.5: 0.5 (v/v) chloroform: methanol: ammonium hydroxide (NH_4OH) at t = 0 min to 60: 34: 5.5: 0.5 (v/v) chloroform: methanol: water: ammonium hydroxide (NH_4OH) at t = 22 min and the column was allowed to equilibrate until the next injection at t = 27 min [19]. The results were the means of three replicates and expressed in g $100g^{-1}$.

3. Cholesterol

For the egg yolk and egg yolk oil cholesterol analysis the standard method of AOAC 994.10 was used with some

modifications. 10 g of the samples were transferred to a 250 ml flask, then 40 ml of ethanol-methanol-isopropanol (90:5:5) solution and 10 ml 60% KOH were added. The flask was connected to the water-cooled condenser and refluxed for 1 hr. After cooling the mixture to room temperature, 100 ml of hexane were added and the mixture was stirred for 10 min. Then 25 ml of deionized water were added and the mixture stirred for a further 15 min. After the layers were separated, hexane layer was collected in an Erlenmeyer flask. An aliquot of 25 ml from the hexane layer was evaporated in a rotary evaporator at 40 °C. The residue was dissolved in 2 ml of ethanol and 3 µl were injected into a gas chromatography [20]. For analysis Shimadzu GC 2010 Plus with flame ionization detector was used. GC conditions: column DB-5 (30 m × 0.32 mm × 0.25 µm), carrier gas: nitrogen, constant flow 0.45 ml min^{-1}, temperature program: 260°C, 6°C min^{-1}, 290°C (8 min), injector: 300°C, split 1:1, detector (FID): 300°C. The results were the means of three replicates and expressed in mg $100g^{-1}$.

4. Carotenoids

1 g of egg yolk or egg yolk oil was accurately weighed in 50 ml glass vial and 5 ml of methanol was added. The sample with methanol was homogenized and then left overnight (16 h) in a refrigerator at 4 °C. Then the sample was centrifugated 800 × g for 10 min. The methanol layer was transferred to a 25 ml volumetric flask. 5 ml tetrahydrofuran (THF) was added to the glass vial with sample and vial was vortexed for 30 seconds, then centrifugated at 800 × g for 5 min. The THF layer was transferred into the methanol containing volumetric flask. The sample was extracted three more times and the THF layers were combined into the volumetric flask. THF was added to make the final volume 25 ml. 10 ml of extract was dried under nitrogen. The extract was resuspended in 500 ml of ethanol and vortexed for 30 seconds. 20 µl were injected into the HPLC system for carotenoid analysis [14].

The carotenoids were determined using a C30 column (3 µl, 150 mm × 4.6 mm, YMC). All carotenoids were monitored at 445 nm with Shimadzu SPD-M20A photodiode array detector. The mobile phase was methanol:tert-Butyl methyl ether:water (95:3:2, v/v, with 1.5% ammonium acetate in water) - solvent A) and methanol:tert-Butyl methyl ether:water (8:90:2, v/v, with 1.0% ammonium acetate in water) - solvent B. The flow rate was set at 0.4 ml min^{-1} (10 °C). Gradient procedure: start at 100% solvent A; a 21-min linear gradient to 45% solvent A and 55% solvent B; 1-min hold at 45% solvent A and 55% solvent B; an 11-min linear gradient to 5% solvent A and 95% solvent B; a 4-min hold at 5% solvent A and 95% solvent B; a 2-min linear gradient back to 100% solvent A, and a 28-min hold at 100% solvent A [14], [21]. Peak identification in samples was based on comparisons with retention time and absorption spectra of known carotenoid standards (lutein, zeaxanthin, canthaxanthin and b-carotene from Sigma-Aldrich Germany). Carotenoids were quantified by integrating peak areas in the HPLC chromatograms. The results were expressed in mg $100g^{-1}$.

III. Results and Discussions

First of all total lipid content of egg yolk, used for egg yolk oil extraction, was measured. Total lipid content of egg yolk sample was 28.34±0.24 g 100 g^{-1}. The content of total lipids in egg yolk is approximately 29% and it does not depend from hens breed and feeding features [22]. The content of total egg yolk lipids was determined to calculate phospholipids, cholesterol and carotenoids content in 100 g of yolk lipids for future loss analysis of these compounds in extracted egg yolk oil. The content of total phospholipids, cholesterol and carotenoids in egg yolk and egg yolk oil is mentioned in Table I.

Because PC and PE are the major egg yolk phospholipids, for determination of distribution of phospholipids in egg yolk oil extraction process these two were taken.

PC and PE content in egg yolk sample was 7.820±0.140 g $100g^{-1}$ and 1.622±0.090 g $100g^{-1}$, respectively. Cholesterol content in egg yolk sample used in this research was 1011 mg $100g^{-1}$.

The content of some bioactive compounds in egg yolk is related to their content in hens' feed [23]. The same is with carotenoids which cannot be sensitized by hens and must be received with diet. Four main carotenoids: lutein, zeaxanthin, canthaxanthin and β-carotene were found in egg yolk (Table I). Lutein was the dominant from all carotenoids present in egg yolk and its content in yolk sample was 0.83±0.02 mg $100g^{-1}$. In other studies lutein also was found as a main pigment of egg yolk where its content in egg yolk was ranged from 0.27 to 1.07 mg $100g^{-1}$ and depended on the method of breeding [24]. Zeaxanthin was also found in egg yolk. In other studies lutein/zeaxanthin ratio in egg yolk was 1:1 [14]. In our study zeaxanthin content in egg yolk was at level 0.42±0.01 mg $100g^{-1}$, that is 2:1 lutein/zeaxanthin. The third carotenoid representing carotenoids in egg yolk was canthaxanthin. Canthaxanthin, same as any other carotenoids, reaches egg yolk from hens feed. Canthaxanthin is added to hens' feed as feed additive – colorant. The dosage of canthaxanthin is strictly regulated by law. The European Unit limit is 8 mg/kg in feed for egg laying hens [25]. Our egg yolk sample contained 0.53±0.01 mg $100g^{-1}$ and the color of yolk was bright orange, which indirectly shows the presence of canthaxanthin. The least carotenoid in egg yolk was β-carotene whose content in egg yolk was only 0.11±0.01 mg $100g^{-1}$. β-carotene is a precursor of vitamin A (provitamin A), the low content of β-carotene in yolk is related, probably, to its fast transition to an active form of vitamin A.

The content of phospholipids, cholesterol and carotenoids in egg yolk oil was determined (Table I). For analysis of extraction loses of these bioactive compounds, the content of phospholipids, cholesterol and carotenoids in total egg yolk lipids was calculated.

Phospholipids content in egg yolk oil was minimal, 0.582±0.009 g $100g^{-1}$ of PC and 0.011±0.009 g $100g^{-1}$ of PE, which was 2.11% and 0.19% from total PC and PE content available in egg yolk lipids (Table II). This result corresponds with [26], where majority of phospholipids was extracted to ethanol extract. Extraction with ethanol with further

purification with acetone is the main method of production of egg yolk lecithin [4] therefore such concentration of PC and PE in ethanol extract was predictable.

TABLE I
PHOSPHOLIPIDS, CHOLESTEROL AND CAROTENOIDS COMPOSITION OF EGG YOLK AND EGG YOLK OIL

	Yolk	Yolk lipids (calculated)	Egg yolk oil
Total lipids,%	28.34±0.24	100	-
PC, g $100g^{-1}$	7.820±0.140	27.59	0.582±0.009
PE, g $100g^{-1}$	1.622± 0.090	5.72	0.011±0.002
PC+PE, g $100g^{-1}$	9.442	33.32	0.593
Cholesterol, mg $100g^{-1}$	1011±10	3567	3105±24
Lutein, mg $100g^{-1}$	0.83±0.02	2.93	0.33±0.07
Zeaxanthin, mg $100g^{-1}$	0.42±0.01	1.48	0.13±0.01
Canthaxanthin, mg $100g^{-1}$	0.53±0.01	1.87	1.68±0.02
β-carotene, mg $100g^{-1}$	0.11±0.01	0.39	0.38±0.01

TABLE II
DISTRIBUTION OF PHOSPHOLIPIDS AND CHOLESTEROL IN ETHANOL AND HEXANE EXTRACTS

	Ethanol extract	Hexane extract following ethanol extraction
PC	97.89%	2.11%
PE	99.81%	0.19%
PC + PE	98.22%	1.78%
Cholesterol	12.95%	87.05%

Distribution of cholesterol within ethanol and hexane extracts, conversely, was unexpected. The biggest part of cholesterol molecule is non-polar, therefore it must be extracted to hexane [27]. But presence of phospholipids in mixed extracts affected the transition of the part of cholesterol into ethanol extract. Many studies report phospholipids' ability to bind cholesterol [5] that can be explained by emulsification properties of phospholipids. In this specific case the part of cholesterol can be captured by phospholipids and transferred into ethanol/water phase when both ethanol and hexane extracts were mixed together [28]. With the presence of water free yolk lecithin can swell and lock some part of cholesterol. But only small part, 12.95% from total available in egg yolk lipids, of cholesterol was transferred into ethanol extract. The other part, not bounded by phospholipids, was free cholesterol which was dissolved in hexane. To remove cholesterol and neutral lipids from phospholipids fraction, repeated extraction with hexane or acetone is used, but still some amounts of cholesterol will be present in purified egg yolk lecithin [4]. As a result of partial cholesterol transition into ethanol extract, its content in egg yolk oil was 3105 mg $100g^{-1}$ or 87.05% from total available in egg yolk lipids.

Behavior of carotenoids in two solvent system is related with its polarity and degree of solubility in particular solvents [13]. Egg yolk carotenoid distribution between ethanol and hexane extracts is summarized is Table III. The majority of lutein and zeaxanthin, as most polar carotenoids presented in egg yolk, was extracted into ethanol/water phase. This is also related to the solubility of these compounds in ethanol and hexane. For example, lutein solubility in ethanol is ten times less than in hexane [17]. It can be explained by the presence of two polar hydroxyl groups in the molecules of lutein and zeaxanthin. Lutein and zeaxanthin concentration in ethanol extract was 88.74% and 91.22% respectively from total available in egg yolk lipids.

Canthaxanthin also is a polar carotenoid, ketocarotenoid containing two carbonyl groups, but it is insoluble in ethanol and water. Solubility of canthaxanthin in hexane is limited [13]. In case of ethanol/water/hexane solvent system canthaxanthin choose to be concentrated in hexane. Canthaxanthin concentration in hexane extract was 89.84% from total content available in egg yolk lipids. Insignificant part of canthaxanthin in ethanol extract can be explained by its inclusion into emulsion made by phospholipids.

97.44% from total egg yolk β-carotene was extracted in to hexane extract. β-carotene is the most non-polar carotenoid from all egg yolk carotenoids, therefore almost all of its content was extracted in to non-polar hexane. As a result, β-carotene, together with canthaxanthin, became major pigments of egg yolk oil.

Lutein is the most important egg yolk carotenoid, because it plays an important role in prevention of age-related macular degeneration [29], therefore decreasing of lutein content in egg yolk oil has a negative effect. But considering that bioavailability of egg yolk lutein is 3 times greater than from a vegetable source [21], egg yolk oil can be still a good source of lutein. The content of all carotenoids in egg yolk oil is presented in Table I.

TABLE III
DISTRIBUTION OF CAROTENOIDS IN ETHANOL AND HEXANE EXTRACTS

	Ethanol extract	Hexane extract following ethanol extraction
Lutein	88.74%	11.26%
Zeaxanthin	91.22%	8.78%
Canthaxanthin	10.16%	89.84%
β-carotene	2.56%	97.44%

IV. CONCLUSION

Distribution of phospholipids, cholesterol and carotenoids during egg yolk oil extraction from liquid egg yolk with two-step extraction using ethanol and hexane was studied. The major amounts of total egg yolk phospholipids (97.89% of PC and 99.81% of PE) were extracted into polar ethanol phase. Cholesterol distribution among ethanol and hexane extracts was 12.95% and 87.05% respectively, which caused the reduction of cholesterol content in egg yolk oil from total cholesterol content presented in egg yolk. Behavior of egg yolk carotenoids was more logical, where more polar lutein and zeaxanthin pass to a polar ethanol extract (88.74% and 91.22% respectively) and non-polar b-carotene (97.44%) to non-polar hexane extract. Canthaxanthin, due its insolubility in ethanol and water (polar extraction phase), choose hexane extract and became a major pigment of egg yolk oil.

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