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Extraction, Evaluation and Comparison of Dried Egg Yolk Phospholipids From Two Different Species of Birds

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EGG yolk phospholipids have a great interest in medical and food medical and food industry due to their biophysical and biological biophysical and biological characteristics. The present study extracts, crystallizes and evaluates the egg yolk phospholipids belonging two different species of birds, hen and duck. Phospholipids were extracted by solvent extraction technique, purified with silica gel, and crystallized by slow evaporation method. Egg yolk phospholipids were characterized by using high pressure liquid chromatography (HPLC), XRD, FT-IR, SEM and TEM spectroscopy. Extracted phospholipids exhibited high purity and identical chemical groups but XRD, HPLC, and TEM results showed significant differences between hen and duck egg yolk phospholipids. Therefore, these results confirm that the species of birds has a more significant effect on the composition and biophysical characteristics of extracted egg yolk phospholipids, emphasizing the importance to control individual parameters for successful using of phospholipids for their specific applications as liposomes for drug delivery.

Keywords: Phospholipids, Hen, Duck, XRD, HPLC, TEM.

In the last decades, phospholipids have become interested natural compounds for various applications including emulsifiers in drug delivery or cosmetics applications, and as nutraceutical ingredients in food industry[(1)]. They have exhibited excellent biological properties such as non-toxicity, biocompatibility, and biodegradability[(2,3)]. For living cells, phospholipids, polar lipids containing phosphate group are the main constitutes in cell membranes forming a bilayer structure that determine the biophysical properties of the cell membrane where it works as electrical insulator surrounding the cells and as signal transmission messengers in addition to carry embedding proteins for cell signalling[(4-6)]. Phospholipids can be extracted from natural sources such as egg yolk, soybean oil, or bacteria[(7-9)]. Egg yolk is rich of biological active compounds such as proteins, phospholipids, vitamins, and antibody[(10-12)]. Egg yolk is considered as one of the most common natural sources for extracting phospholipids which represent about 15% of dried egg yolk powder. Phosphatidylcholine and phosphatidylethanolamine are the two main phospholipids representing roughly about 70% and 20% of phosphatidylethanolamine of total weight of egg yolk

phospholipids[13]. Various methods and techniques based on solvent systems have been used for extracting egg yolk phospholipids[14]. All of these methods and techniques are influenced by various factors such as solubility variations of egg yolk components in water and organic solvents, different ratios of polar and nonpolar lipids, temperatures and pH values[15-18]. The phospholipids are still commonly used for specific pharmaceutical products and drug delivery systems by preparing liposomes[19]. The liposomes are lamellar structures that formed when dried phospholipids such as phosphatidylcholine (PC) have exposed to excess aqueous media. In aqueous solutions, phospholipids are not soluble and align themselves closely but they are soluble in organic solvents[20]. Selection of the phospholipids depending on their purity, nontoxicity and sources is the first step in preparing the liposomes. Various studies have reported that the characteristics of liposomes, *i.e.* their sizes, stability and interactions with loaded drugs, depends strongly on the phospholipids compositions. Also, the characteristics of dry phospholipids such as their surface area, porosity, and particles size are of particular importance for prepared liposomes[21]. Drying processes of phospholipids have proceeded from their volatile organic solutions by using slow evaporation, spray drying, and lyophilization[22]. Dried phospholipids extracted from egg yolk have advantages compared with that extracted from soybeans where they have a higher proportion of fully saturated fatty acyl chains, and are rich of choline that is important for developing and function of nervous system[23-25]. However, information on dried phospholipids crystals is not available. Dried phospholipids crystals have important impacts, mainly for designing and preparing of liposomes whose biophysical properties depend largely on the presence crystals. Egg yolk belonging various strains or species of birds such as chicken, duck or turkey has significant differences in phospholipids compositions and concentrations[26]. Considering the importance of egg yolk phospholipids, the aim of this study is to extract, crystallize and compare the biophysical characteristics of the dried egg yolk phospholipids extracted from two different species of birds, hen and duck. Therefore, HPLC was used to test the purity of extracted phospholipids. The crystallization behaviour was confirmed by XRD technique, while the morphology and microstructure were characterized by SEM and TEM spectroscopy. FT-IR spectroscopy provided information on the functional chemical groups of extracted phospholipids.

Experimental Methods

Extraction, purification and crystallization egg yolk phospholipids

Five of fresh eggs of domestic hen (solid white *Gallus gallus domesticus*) and duck (*Anas platyrhynchos*), were obtained from a local poultry farm in Tanta, El-Gharbia Province, Egypt. 100 g of hen and duck egg yolk were mixed with water of pH = 5.0 using sample mass/solvent volume ratio (1:9). The suspension was centrifuged to separate insoluble precipitated components from the supernatant. The precipitate was dissolved in absolute ethyl alcohol using sample mass/solvent volume ratio (1:8) then the solution was centrifuged to separate the supernatant. To purify the crude phospholipids, the supernatant from previous

step was eluted in silica gel column chromatography, radius 3 cm and height 30 cm, by using acetone/hexane volume ratio (9:1). Charcoal and sodium sulphat anhydrous were added to the extracted solution obtained from the colomn chromatography and filtered using filter paper. To crystallize extracted phospholipids, the phospholipids were dissolved in 50 ml hexane in a 250 ml beaker and the solvent was allowed to evaporate slowly over 3-4 d.

Characterization of dried egg yolk phospholipids

HPLC analysis of extracted dried egg yolk phospholipids

The purity of the samples is checked by using Agilent 1100 series HPLC instrument. Extracted hen and duck phospholipids purified by silica gel column chromatography were measured. All samples were eluted by methanol within 11 min. 402 and 460μg of hen and duck egg yolk phospholipids with concentration of 357 and 436μg/μl, and an injection volume of 20 and 10 μl at 30°C, respectively were used for testing. Chromatographic peaks were identified by comparing with PL standards and purity of the target species were calculated as the percentage of total PL peak area. A 13.5μg of standard phosphatidylcholine with concentration of 1.35μg/μl was used for comparison and calibration.

X-ray diffraction of extracted dried egg yolk phospholipids

The structure of dried egg yolk phospholipids extracted from hen and duck egg yolks were analyzed by XRD at room temperature. Powder XRD patterns of the samples were recorded using a GNR- APD 20000 pro, H423-vertical diffractometer in the range (2θ from 5° to 80°) under the following conditions: 40 kV, 30 mA, Cu-Kα radiation (λ = 0.15406 nm). The samples were scanned in steps of 2◦ using a count time of 4 s per step. The crystallite size broadening of a diffraction peak can be related to the mean crystallite size via the Scherer equation, $t = (0.9 \cdot \lambda)/(B \cdot \cos\theta_B)$, where t is the mean crystallite size, B is the peak line-width at half maximum (in radian), θ_B is the Bragg diffraction angle and λ is the X-ray (CuKα radiation) wavelength.

FT-IR spectroscopy of dried egg yolk phospholipids

FT-IR was employed to identify the IR active covalent functional groups and structure of dried egg yolk phospholipids extracted from hen and duck egg yolk. FT-IR spectra were recorded on a Bruker Tensor 27 at room temperature. The samples were mixed with dried KBr at ratio 1:10, respectively then pressed into a disc. The FT-IR spectra for each sample were taken in the region from 400 cm^{-1} to 4000 cm^{-1}.

Surface topography and morphology of dried egg yolk phospholipids

A scanning electron microscope was employed to examine the surface topography and formation of dried egg yolk phospholipids extracted from hen and duck egg yolk. SEM images were obtained with a JXA-840A electron microscope equipped with a backscatter electron detector. The measurements were taken at 15kV of electron acceleration voltage. The transmission electron microscope is generally used to characterize the microstructure and morphology

of extracted samples. TEM images were recorded on a JEOL-JEM-2100 operating at an acceleration voltage of 80 kV. The specimens for TEM investigations were prepared by placing a drop of each sample suspension on a carbon-coated copper grid and allowing this to dry in air under ambient conditions.

Results and Discussion

Purity and composition analysis

HPLC results of standard phosphatidylcholine (PC) and phospholipids extracted from hen and duck egg yolk are shown in Fig.1. Refined hen and duck egg yolk phospholipids were identified by comparing with the retention time of standard PC. Fig.1a shows a typical chromatogram of standard PC with pure perfect single peak at elution time of 6.3 min. Fig.1 (b and c) shows high purity of 93 and 94% of extracted hen and duck egg yolk phospholipids, respectively. The chromatogram profiles exhibit significant differences between the phospholipids extracted from hen and duck egg yolk at retention time of 6.34 and 6.38, respectively. The profile, shape and multi-peaks of the two sample confirming presence a different types of phospholipids with PC having complex fatty acid residues[15,27]. The results show a better separation of hen egg yolk phospholipids than duck egg yolk phospholipids. This difference can be attributed to the variation between concentration and classes of phospholipids composing hen and duck egg yolk.

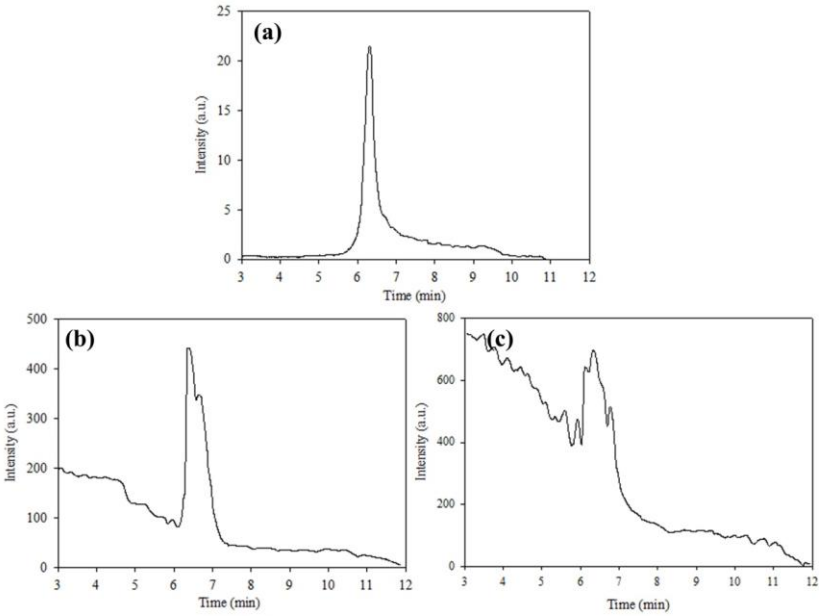

Fig. 1. Chromatogram of the standard PC (a), hen egg yolk phospholipids (b), duck egg yolk phospholipids and (c) (Mobile phase, methanol; flow rate, 1.0 ml·min^{-1}; column temperature, 30 °C; sample injection, 10, 20 and 40 µl, respectively).

Phase analysis

The XRD patterns of phospholipids samples obtained after evaporation of solvent are shown in Fig.2. XRD patterns have shown that hen egg yolk phospholipids have exhibited crystalline phase with main peak at 2theta = 19.4° and several sharp peaks at 13.2, 15, 16.2, 24.2, 26.2, 29.5, 40.3, and 43.1°, Fig.2(a). XRD pattern for duck egg yolk phospholipids exhibited poorly crystalline phospholipids phase represented by a broad diffraction pattern at 2theta = 20°, Fig.2(b), indicating presence of amorphous structures where a part of the extracted amorphous phospholipids do not convert to crystalline phase. The observed variations in XRD of hen strain and duck egg yolk phospholipids are mainly attributed to the difference in their species. This significant variation in the crystalline and amorphous composition is mainly due to the different compositions of phospholipids. These results indicate that the hen egg yolk phospholipids powder is higher crystalline than that of duck egg yolk phospholipids powder. The crystallinity of solvent-extracted phospholipids nanoparticles plays a role in fabrication of drug carriers. The changes of the crystallinity mean changes in the interaction and entrapment of drugs within phospholipids layers[28]. Using phospholipids as carriers for lipophilic drugs can enhance their solubility and bioavailability[29]. However, Most studies used XRD to evaluate the crystallinity of solvent-extracted phospholipids indicated their amorphous structures[30-32].

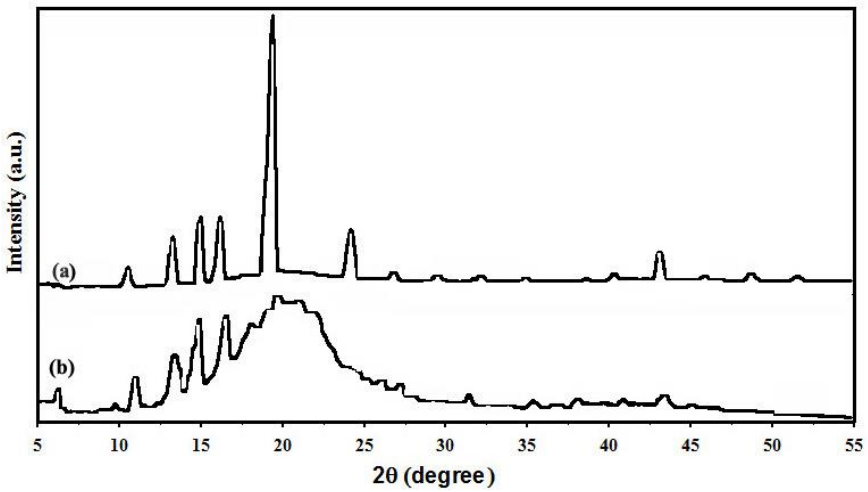

Fig. 2. X-ray diffraction peaks of dried extracted hen egg yolk phospholipids (a) and duck egg yolk phospholipids (b).

FT-IR analysis

The FT-IR spectra, Fig. 3 (a and b) clearly demonstrate the major peaks associated with extracted phospholipids. All extracted phospholipids are identical and exhibited the same characteristic absorption bands indicating that the samples have the same functional chemical groups. The hen and duck egg

yolk phospholipids exhibited characteristic vibrational symmetric stretching modes of the methylene groups of the fatty acid contained in phospholipids, $-CH_3$ at 1450 and 2830 cm^{-1}, The $-CH_2$ asymmetric stretching band at 1350 and 2903 cm^{-1}. A strong absorption band at 1720 cm^{-1} supports the presence of the vibrational stretching mode of C=O, ester carbonyl. The phosphate group characterized the phospholipids exhibits $-PO^{-2}$ asymmetric stretching bands at 1080 and 1220 cm^{-1}. The absorption bands at 1030 and 3440 cm^{-1} are attributed to the ($^{+}N(CH_3)$) asymmetric stretching band and the absorption band of water , respectively.

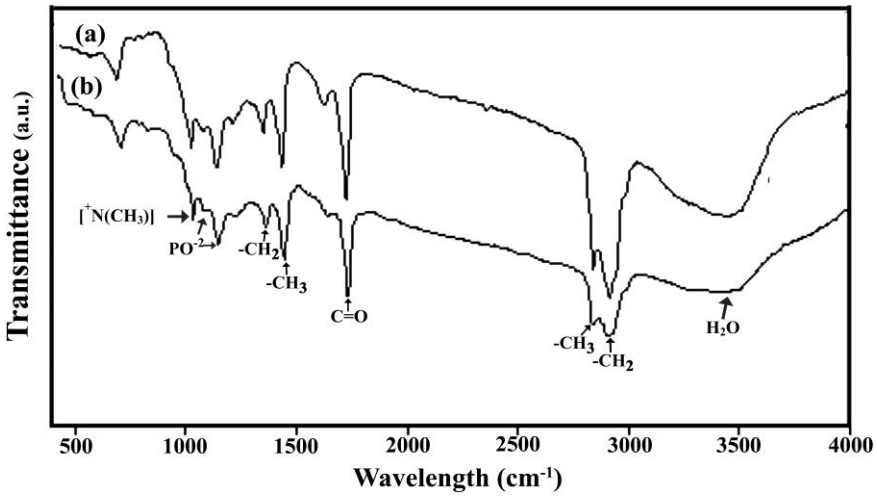

Fig. 3. FT-IR spectra of dried extracted hen egg yolk phospholipids (a) and duck egg yolk phospholipids (b).

Surface topography and morphology analysis

Figure 4 (a and b) demonstrated the SEM images of extracted hen and duck egg yolk phospholipids powders, respectively. Crystallized phospholipids have external smooth surface having an irregular rippled texture. The surface ripples appear to have a directional grooves with absence of microscale features indicating high tightly ordered phospholipids particles bonding together in which the dried phospholipids existed in the crystal state. Figure 5 showed the particle size and appearance of extracted hen and duck egg yolk phospholipids. All phospholipids powders have nanosized particles with spherical morphology and sizes at 30 nm and 20 nm for hen and duck egg yolk phospholipids, respectively as shown in Fig.5 (a and b). Phospholipids particles from the two samples exhibited highly agglomeration due to the Van der Waals forces between nanoparticles. Phospholipids extracted from hen showed larger particle size than that of duck.

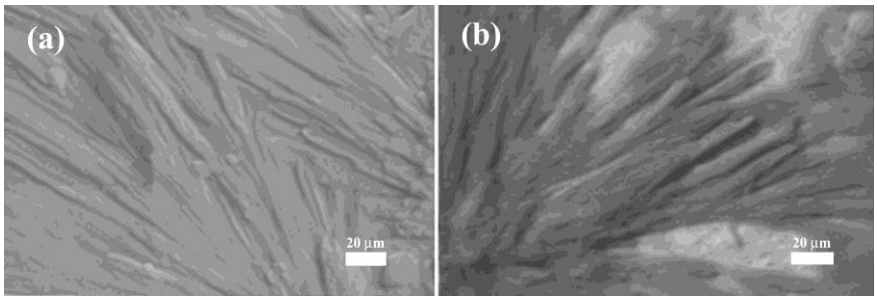

Fig. 4. SEM images of the surfaces of (a) dried extracted hen egg yolk phospholipids and (b) duck egg yolk phospholipids.

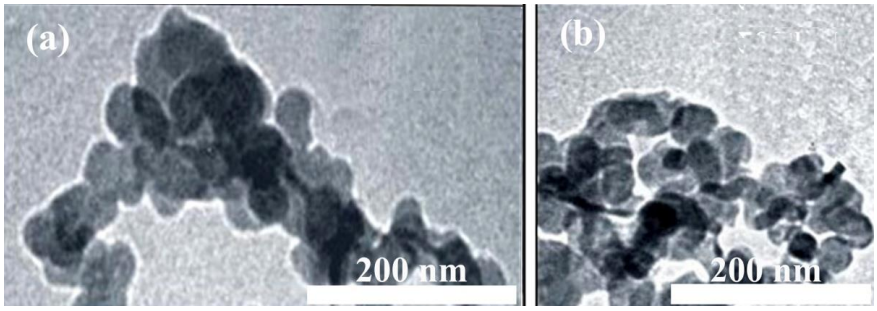

Fig. 5. TEM images of the morphology of (a) extracted hen egg yolk phospholipids and (b) duck egg yolk phospholipids.

Conclusion

Pure egg yolk Phospholipids of two different species of birds, hen and duck, were extracted and crystallized. The extracted phospholipids composition has exhibited significant differences between hen and duck egg yolk. The hen egg yolk phospholipids exhibited more crystalline state than duck egg yolk phospholipids indicating variation of phospholipids compositions. TEM spectroscopy provided a direct method to detect differences between sizes of agglomertated particles size of hen and duck egg yolk phospholipids. The results of the present study demonstrate significant differences of biophysical properties between hen and duck egg yolk phospholipids that are valuable and essential to improve the pharmaceutical products depending on using egg yolk phospholipids.

References

1. **Martiel, I., Sagalowicz, L. and Mezzenga, R.,** Phospholipid-based nonlamellar mesophases for delivery systems: Bridging the gap between empirical and rational design: Review. *Advances in Colloid and Interface Science*, **209**,127-143 (2014).

2. **Jena, S.K., Singh, C., Dora, C.P. and Suresh, S.** Development of tamoxifen-phospholipid complex: Novel approach for improving solubility and bioavailability. *International Journal of Pharmaceutics*, **473**, 1-9 (2014).

3. **Meenach, K.W., Anderson, J.Z., Hilt, R.C. and McGarry, H.M.** Mansour. Characterization and aerosol dispersion performance of advanced spray-dried chemotherapeutic PEGylated phospholipid particles for dry powder inhalation delivery in lung cancer. *European Journal of Pharmaceutical Sciences*, **49**, 699-711 (2013).

4. **Tan, D., Zhang, X., Li, J., Tan, H. and Fu., Q.** Double-chain phospholipid end-capped polyurethanes: Synthesis, characterization and platelet adhesion study. *Applied Surface Science*, **258**, 2697-2706 (2012).

5. **Morita, S., Umakoshi, H. and Kuboi, R.** Dielectric response of cells and liposomes and its utilization for evaluation of cell membrane-protein interaction. *Journal of Bioscience and Bioengineering*, **90**,157-162 (2000).

6. **Xiang, S.Y., Dusaban, S.S. and Brown, J.H.** Lysophospholipid receptor activation of RhoA and lipid signaling pathways. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, **1831**, 213-222 (2013).

7. **Gładkowski, W., Kiełbowicz, G. A., Chojnacka, M., Gil, T., Trziszka, Z. Dobrzański and Wawrzeńczyk, C.** Fatty acid composition of egg yolk phospholipid fractions following feed supplementation of Lohmann Brown hens with humic-fat preparations. *Food Chemistry*, **126**, 1013-1018 (2011).

8. **Navari-Izzo, F., Vangioni, N. and Quartacci, M.F.** Lipids of soybean and sunflower seedlings grown under drought conditions. *Phytochemistry*, **29**, 2119-2123 (1990).

9. **Gidden, J., Denson, J., Liyanage, R., Ivey, D.M. and Lay Jr. J.O.** Lipid compositions in *Escherichia coli* and *Bacillus subtilis* during growth as determined by MALDI-TOF and TOF/TOF mass spectrometry. *International Journal of Mass Spectrometry*, **283**, 178-184 (2009).

10. **Byrne, B.M., Gruber, M. and Ab., G.** The evolution of egg yolk proteins: Review. *Progress in Biophysics and Molecular Biology*, **53**, 33-69 (1989).

11. **Devi, C.M., Bai, M.V. and Krishnan, L.K.** Development of viper-venom antibodies in chicken egg yolk and assay of their antigen binding capacity. *Toxicon*, **40**, 857-861 (2002).

12. **Fukuzawa, K. Ouchi, A., Shibata, A., Nagaoka, S. and Mukai, K.** Kinetic study of aroxyl radical-scavenging action of vitamin E in membranes of egg yolk phosphatidylcholine vesicles. *Chemistry and Physics of Lipids*, **164**, 205-210 (2011).

13. **Burley, R.W. and Vadehra, D.V.** "*The Avian Egg Chemistry and Biology*", John Wiley & Sons (1989).

14. **Nielsen, H.** Production of phospholipids from spray-dried egg yolk by consecutive *in situ* solid phase extraction with acetone and ethanol. *LWT - Food Science and Technology*, **40**, 1337-1343 (2007).

15. **Yoon, T.H.and Kim, I.H.** Phosphatidylcholine isolation from egg yolk phospholipids by high-performance liquid chromatography. *Journal of Chromatography*, A **949**, 209-216 (2002).

16. **Komatsu, M. and Ando, S.** Isolation of crustacean egg yolk lipoproteins by differential density gradient ultracentrifugation. Comparative Biochemistry and Physiology Part B: *Comparative Biochemistry*, **103,** 363-368 (1992).

17. **Palacios, L.E. and Wang, T.** Extraction of egg-yolk lecithin. *Journal of the American Oil Chemists' Society* **82**, 565-569 (2005).

18. **Ramesh, B., Adkar, S.S., Prabhudesai, A.V. and Viswanathan, C.V.** Selective extraction of phospholipids from egg yolk. *Journal of the American Oil Chemists' Society* **56**, 585-587 (1979).

19. **Constantinides, P.P., Chaubal, M.V. and Shorr, R.** Advances in lipid nanodispersions for parenteral drug delivery and targeting: Review. *Advanced Drug Delivery Reviews* **60**, 757-767 (2008).

20. **Antonietti, M. and Förster, S.** Vesicles and Liposomes: A Self-Assembly Principle Beyond Lipids. *Adv. Mater*., **15** (16), 1323-33 (2003).

21. **Betageri, G.V., Jenkins, S.A. and Parsons, D.L.** "*Liposome Drug Delivery Systems*". Technomic Publishing CO., Inc. Pennsylvania, USA, (1993).

22. **Wessman, P., Edwards, K. and Mahlin, D.** Structural effects caused by spray- and freeze-drying of liposomes and bilayer disks. *Journal of Pharmaceutical Sciences,* 99, 2032–2048 (2010).

23. **Ide, T. and Murata, M.** Depressions by dietary phospholipids of soybean and egg yolk origins of hepatic triacylglycerol and fatty acid synthesis in fasted-refed rats. *Ann Nutr Metab* **38**, 340-8 (1994).

24. **Tocher, D.R., Bendiksen, E.Å. Campbell, P.J. and Bell, J.G.** The role of phospholipids in nutrition and metabolism of teleost fish: Review. *Aquaculture,* **280**, 21-34 (2008).

25. **Vance, J.E. Campenot, R.B. and Vance, D.E.** The synthesis and transport of lipids for axonal growth and nerve regeneration: Review. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, **1486**, 84-96 (2000).

26. **Watson, R.R.** "*Eggs and Health Promotion*". A Blackwell Publishing Company, Iowa State Press (2002).

27. **Jing, Z., Xianmei, H., Tianxin, W., Hao, L. and Qipeng, Y.** HPLC Analysis of Egg yolk phosphatidylcholine by evaporative light scattering detector. *Chinese Journal of Chemical Engineering*, **20**, 665-672 (2012).

28. **Yue, P.F., Zhang, W.J., Yuan, H.L., Yang, M., Zhu, W.F. and Cai, P.L.,** Process optimization, characterization and pharmacokinetic evaluation in rats of ursodeoxycholic acid-phospholipid complex. *AAPS Pharm Sci Tech*, **9**, 322-329 (2008).

29. **Cui, F.D., Shi, K., Zhang, L.Q., Tao, A.J. and Kawashima, Y.** Biodegradable nanoparticles loaded with insulin phospholipid complex for oral delivery: preparation, *in vitro* characterization and *in vivo* evaluation. *J. Control. Release* **114**,242–250 (2006).

30. **Cai, X., Luan, Y., Jiang, Y., Song, A., Shao, W., Li, Z. and Zhao, Z.** Huperzine A–phospholipid complex-loaded biodegradable thermosensitive polymer gel for controlled drug release. *International Journal of Pharmaceutics*, **433**,102-111(2012).

31. **Tessier, C., Nuss, P., Staneva, G. and Wolf, C.** Modification of membrane heterogeneity by antipsychotic drugs: An X-ray diffraction comparative study. *Journal of Colloid and Interface Science*, **320**, 469-475 (2008).

32. **Huang, J., Gou, G., Xue, B., Yan, Q., Sun, Y. and Dong, L.** Preparation and characterization of "dextran-magnetic layered double hydroxide-fluorouracil" targeted liposomes. *International Journal of Pharmaceutics* **450**, 323-330 (2013).

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استخلاص وتقدير ومقارنة فوسفوليبدات مح البيض المجفف لنوعين مختلفين من الطيور

هانى على الغرباوى ،رضا محمد مرسى وطارق محمد النمر
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فسوفوليبدات مح البيض لها أهمية قصوى فى صناعة الغذاء والطب نتيجة خواصها البيولوجية والبيوفيزيائية . تهتم الدراسة الحالية باستخلاص وبلورة وكذلك تقدير فسوفوليبدات مح البيض لنوعين مختلفين من الطيور هما الدجاج والبط . تم استخلاص الفسفوليبدات بواسطة تقنية الاستخلاص من المذيب، وتقنية السيليكا جيل والبلورة عن طريق التبخير البطئ. ثم تم فحص فسفوليبدات مح البيض باستخدام تقنيات كل من مطياف حيود الاشعة السينيه، وجهاز high pressure liquid chromatography(HPLC) ومطياف الاشعة تحت الحمراء بالإضافة إلى الميكروسكوب الالكترونى الماسح و النافذ.

أظهرت هذه الدراسة أن الفسفوليبدات المستخلصه لها نقاوة عالية وتتطابق مع المجموعات الكيميائية المماثلة ولكن أوضحت نتائج حيود الاشعة السينيه و HPLC وكذلك الميكروسكوب الالكترونى النافذ فروقا جوهرية بين فسفوليبدات مح البيض لكل من الدجاج والبط . بناءا على ذلك تأكد هذه النتائج ان نوعية الطيور لها تأثير على كل من التكوين والخصائص البيوفيزيائية لفسفوليبدات مح البيض المستخلصه والتى تأكد اهمية التحكم فى العوامل الذاتيه لاستخدام الفسفوليبدات المناسب للتطبيقات فى إعداد الليبوسومات لنقل الدواء إلى الأماكن المطلوبة .