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CHROMATOGRAPHIC ASSAY OF CHOLESTEROL IN CHICKEN EGGS

Miriam A. ElDeeb[1] **and Samy Emara**[2]

1- Department of Animal Production, Faculty of Agriculture, University of Assiut, Assiut, Egypt,
2- Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, University of Assiut, Assiut, Egypt

SUMMARY

An analytical assay using HPLC equipped with a stainless steel analytical column (150x4.6 mm I.D.) packed manually with ODS/TM silica (pore size, 80 Å) has been optimized for the extraction selectivity and resolution of cholesterol from chicken egg. The identity of cholesterol separated from egg yolk was confirmed using Sigma authentic cholesterol. The assay procedure was found to be rather sensitive and concentration dependent which allowed quantitative determination of cholesterol from several individual samples. The frequency of calculated cholesterol as per g egg yolk fell within the published figures which indicated reproducibility and reliability of the assay methodology. Furthermore, when authentic cholesterol was added to egg yolk and then subjected to the extraction and determination procedures, recovery was more than 99% which indicated that the assayed cholesterol from egg yolk represented the actual value. It is important to note that the analytical procedure developed in this manuscript is precise and allows no future used of internal standards. This facile and modified assay was proven to be highly applicable in studying nutritional effects on chicken-egg cholesterol.

Keywords: Cholesterol assay, HPLC, egg yolk, chromatography

INTRODUCTION

Cholesterol is a polycyclic ring lipid structure that has enormous functions in the biological systems. It constitutes major component of plasma membrane and is the precursor of all bile acids and steroid hormones. Through its hydrophobic interaction with phospholipids and glycolipids in the lipid bilayer of eukaryotic membranes, especially erythrocyte membranes, cholesterol serves to determine membrane fluidity and maintain mechanical stability (Maclean, 1987). The fluidity, stability and the precursory functions of cholesterol indicate the importance of its dietary intake, regulation and metabolism. It also indicates that the complexity of its functions proceeds at risks of inflammation and fibrosis and some other lesions associated with cholesterol stone formation. In particular, excessive intake of lipids may cause arteriosclerosis (Food and Nutrition Board, 1953), probably due to hypercholesterlaemia. Therefore, cholesterol content in animal products, especially those consumed by humans appears to be of critical importance. In this regard, eggs are considered to be a major source of animal protein. Factors affecting egg yolk cholesterol deposition has been widely investigated (Haris, 1988). It is also known that cholesterol metabolism in hens is influenced by several factors including dietary cholesterol, caloric intake and genotype (Sutton et al., 1984). In the Department of Animal Production, Assiut University, we are currently investigating the effects of feeding some natural products of plant origin on the cholesterol level of egg-yolk (ElDeeb, 1994). It was then planned to develop a fast, reliable and reproducible assay to be routinely used in these investigations, especially after the commencement of strong awareness of cholesterol detrimental effects among the Egyptian public.

Several methods have been adopted to assay for cholesterol levels in foodstuffs including egg yolk. These methods were based either on a colorimetric technique (Zlatkis et al., 1953 & Bair and Marion, 1978), gas liquid chromatographic technique (Adams et al., 1986) or High performance liquid chromatography (HPLC) technique (Naber and Biggert, 1985). Most of chromatographic techniques were based on the physical

properties of cholesterol that can be manipulated using organic solvents of different properties. Usually these techniques are tedious, expensive and far from being quantitative. Therefore, these points were taken into consideration in developing the assaying procedure in the present manuscript.

The HPLC system was used to accurately assay for cholesterol contents in egg yolk. As a start, this manuscript dealt with the development of an extraction methodology, which was based on protein precipitation instead of organic extraction to avoid problems associated with quantitative assay using solvent extraction. Several modifications (Emara, 1993) were undertaken to maximize the precision of the developed assay.

MATERIALS AND METHODS

Instrumentation

HPLC apparatus consisted of a model HLC-803 pump (Toyo, Soda, Japan), equipped with Rheodyne model 7125 syringe loading sample injector adjusted to 200-ul loop (Rheodyne, Berkeley, CA, USA). This system was equipped with a stainless steel analytical column (150x4.6 mm ID.) packed manually with ODS/TM silica (pore size, 80 Å, particle size of mu; Toyo, Soda, Japan). The chromatographic detector used was a spectrophotometric detector (UV-8 Model II; Toyo, Soda, Japan), operated at a wavelength of 208 nm. Peak heights were computed using a Shimadzu C-R3A chromatopac integrator (Shimadzu, Kyoto, Japan).

Standardization

Fifty ul aliquot samples of increasing concentrations of cholesterol in acetonitrile was injected into the HPLC column for standardization and the peak height at the retention time of cholesterol was measured. The mobile phase in this HPLC technique was the isocratic solvent system of methanol and 0.1 M of NaH_2PO_4 at 80:20 volumetric ratio. The phosphate solution was adjusted to pH 4.0 using HCl and filtered before mixing with methanol. The mobile phase was daily prepared and carefully degassed before use. A total number of 8 concentrations ranged from 5-40 ug/sample was assayed at six replicates per each concentration. Authentic

cholesterol was from Sigma. The flow rate was 1.2 ml/min and column temperature was 25°C.

Sample Preparation and Application

Eggs were obtained from a commercial layers, single comb white Leghorn hens (Hy-Line), that were raised in the Farm of the Department of Animal Production, Assiut University. Two gram sample of egg yolk weighed and quantitatively transferred to a 10-ml volumetric flask containing 4 ml of 0.05 M NaOH. The sample was mixed gently and completed to the final volume of 10 ml using 0.05 M HCl and thoroughly mixed. Two ml was then added to an equal volume of acetonitrile in a 15-ml centrifuge tube and the mixture was then shaken vigorously before centrifugation at 2000 rpm for 5 minutes and the homogenous supernatant was filtered through a 0.45 mu membrane filter unit, Millex-GS (provided by Millipore, Bedford, Mass., USA). This filtration process assured analyses of egg yolk cholesterol with no complication due fluctuation in the column pressure. Fifty ul aliquots of the acetonitrile layer was then injected into the HPLC column for sample assay under conditions similar to those of the standard cholesterol. The peak height of egg-yolk cholesterol was measured and the corresponding concentration was then computed from the standard curve regression equation.

All the glassware used in the present study was silanized and rinsed with 3% dichloromethyl silane in toluene and methanol, respectively. Also all solvents used in this manuscript were HPLC reagent grade.

RESULTS AND DISCUSSION

The chicken eggs have always been considered by the Egyptian community as an important part of the diet. However, the recent international concern bring awareness of the cholesterol detrimental effects and this awareness has given eggs a bad rap. Due to the lack of baseline data on cholesterol of Egyptian chickens from different breeds and under different environmental and management conditions, it is important to develop an assay for cholesterol content to possibly study cholesterol in egg yolk and monitor any change in the baseline data in eggs absorbed by the Egyptian market. This information will be instrumental not only at the

applied research level but at the academic level, when factors affecting cholesterol in chicken eggs are to be studied. Due to the importance of cholesterol determination, an HPLC assay was developed based on the modification of the published procedures. The extraction selectivity and resolution efficacy were optimized in preliminary experiments to minimize interference between cholesterol and endogenous polar compounds. This was achieved by optimizing pH, salt concentration, types and concentrations of organic modifiers. The technique has proven successful in clear resolution of egg yolk cholesterol (Figure 1). Six random samples each of authentic and egg yolk cholesterol were subjected to the assay procedure and the mean retention times were 8.115 and 8.125 min, respectively. This further adds experimental evidence for the identity of the assayed cholesterol from egg yolk.

After optimization, a series of cholesterol concentrations (5-40 μg) were then used for the preparation of a standard curve (Figure 2). As shown in this figure, the relationship between the concentration of cholesterol and the peak height following HPLC separation and detection seems to be both qualitative and quantitative. The regression equation from which unknown samples were assayed for their cholesterol content is shown below:

Peak height= 1.2 x cholesterol conc (μg) - 0.034 (1)
where peak height is expressed in integrator's counts/1000.

As cholesterol is one of many polyring lipids, it was decided to check for the recovery of authentic cholesterol when added to egg yolk and subjected to the extraction procedure. Results in Table 1 indicate clearly that minute, but nonsystematic variation was obtained in the recovery of cholesterol concentrations ranged from 5 to 40 μg per sample. However, the percentage recovery was always higher than 99%.

It is also interesting that peak heights, corresponding to the same cholesterol concentrations which directly chromatographed or chromatographed following mixing with egg yolk and then extracting using the same procedures, exhibit a unique straight line relationship (Figure 3). The enriched peak height was corrected by subtraction of the peak height

corresponding to endogenous cholesterol in the egg-yolk sample with no enrichment.

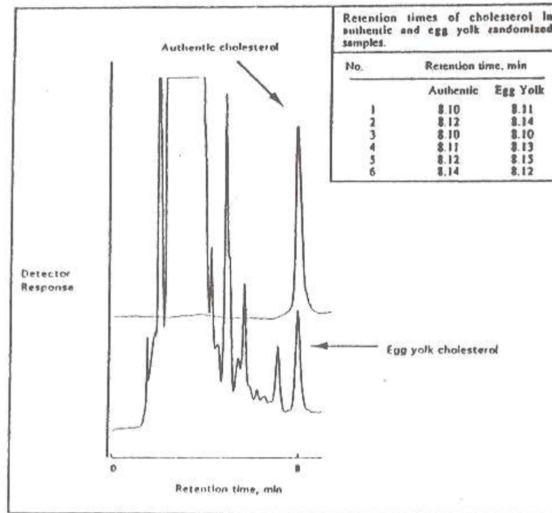
Table 1. Recovery of authentic cholesterol when added to and thoroughly mixed with egg yolk and subjected to the separation and determination procedures developed in the present study

Theoretical (added) ug	Peak height	Assayed, ug*	Recovery %**
5.0	5.97	5.003	100.06
10.0	11.99	10.020	100.20
15.0	17.95	14.986	99.90
20.0	24.00	20.028	100.14
25.0	29.94	24.978	99.91
30.0	35.97	30.003	100.01
35.0	41.96	34.995	99.98
40.0	47.99	40.020	100.05

* Calculated cholesterol concentrations were computed from Equation (1) in the text which related the HPLC peak height to the concentration of cholesterol in ug/sample.

** Mean ± SE = 100.03 ± 0.036

As shown in Figure 3, and its regression equation, it appears that almost absolute recovery was performed under the extraction methodology used for egg-yolk sample cholesterol. The recovery can be easily indicated by the slope of the line which is an almost unity (slope= 0.9989). This further confirms the reliability and reproducibility of the developed methodology in determining cholesterol from egg yolk samples. The intercept on the y-axis may implicitly expresses the interference of other materials in the assay procedure. This intercept is minimal (y-intercept 0.006 mm) which is equivalent to only 0.03 ug cholesterol when equation (1) was used to convert peak height into concentration units. Whether this minute amount is due to recovery reasons or due to an interference from other materials with identical retention time is unclear. However, since the recovery is almost absolute this extremely minor underestimation is insignificant regardless of the interfering factor(s). The method, in general, appears to be ideal for the extraction selectivity and HPLC resolvability of cholesterol.



No.	Retention time, min	
	Authentic	Egg Yolk
1	8.10	8.11
2	8.12	8.14
3	8.10	8.10
4	8.11	8.13
5	8.12	8.15
6	8.14	8.12

Fig. 1. Retention time of extracted chicken egg-yolk cholesterol as compared to authentic Sigma standard following HPLC assay. Inset indicates the retention times of egg-yolk and authentic cholesterol in twelve random runs.

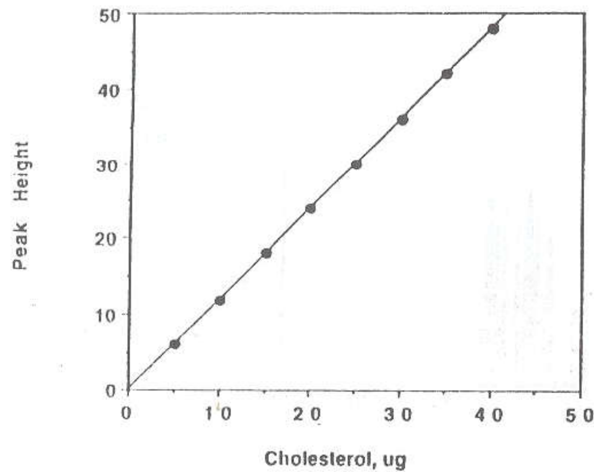


Fig. 2. Relationship between concentrations of authentic cholesterol from Sigma and the corresponding peak heights (expressed as integrator's counts/1000) following HPLC assay.

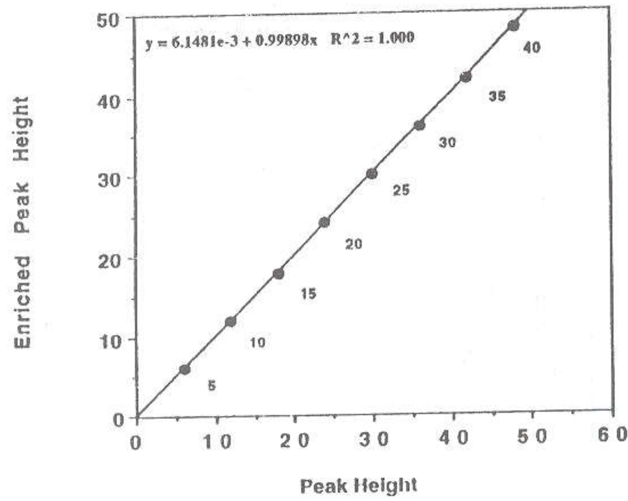


Fig. 3. Relation between the peak heights of increasing concentrations of Sigma authentic cholesterol (numbers next to datum points are ug cholesterol injected/standard sample) when directly injected into HLPC system or when added to egg-yolk and subjected to the extraction procedure prior to injection.

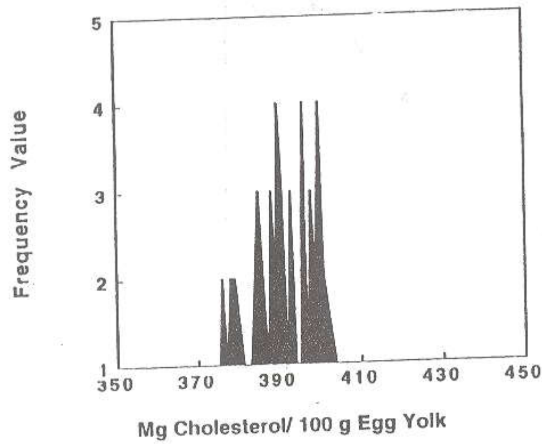


Fig. 4. Frequency of cholesterol in chicken eggs. A total number of 56 eggs was individually extracted and HLPC-assayed for their cholesterol content.

After recognizing the identity of measured cholesterol in egg-yolk (Figure 1) and the quantitative extraction and resolvability of the cholesterol content in egg-yolk, 128 eggs (from the same source) were individually assayed for their cholesterol content. The results of this experiment are shown in Figure (4).

As shown in this figure, it appears that cholesterol in chicken egg-yolk falls with a reasonable limit (about 374-400 mg/100 g yolk). This distribution is consistent with previous findings indicating the presence of sufficient genetic variability in yolk cholesterol levels (Hargis, 1988). The frequency distribution, as well as the overall mean (390 mg/100 egg yolk) conclusively confirms the validity of the optimized HPLC assay methodology. Recent publication reported an overall average of egg-yolk cholesterol from Hy-Line to be 364 mg/100 g (Sloan et al., 1994). Our reported values are reasonably ranged within those of other published reports which confirms the overall precision and subsequent reproducibility of the developed techniques. It is, therefore, recommended that this procedure is to be routinely used in poultry science laboratories for physiological and/or nutritional experiments. For example, the data base of cholesterol levels will allow us to study the environmental, management and genetic factors affecting cholesterol level. This study would be of special merit if one can balance the level of cholesterol so that its embryonic function is secured without any undesired effects when consumed by humans.

CONCLUSION

The importance of cholesterol assay lies in the multifunction role of this steroid congener in the biological system. Cholesterol is a component of all the body cells of humans and animals. It is needed to form hormones, cell membranes, and other body substances. Contrary to food of plant origin, cholesterol is present in all animal products, e.g., poultry, fish, milk and milk products and egg-yolk. Also it is found in mixtures such as baked products and mayonnaise that contain egg-yolks, cheese, milk-butter, or lard as ingredients. It is now a common belief that human diet low in saturated fat and cholesterol can help maintaining a desirable

cholesterol level and reduce the risk of heart diseases. Therefore, awareness of the magnitude of cholesterol in foodstuffs is increasing among people from developed and developing countries. The commencement of this awareness among Egyptian public has been very strong and raises many concerns. Obviously, there is always need for reliable, reproducible and simple assay of cholesterol in food products of animal origin. In this manuscript, we have tried to develop a chromatographic method to assay for cholesterol in chicken egg-yolk. The analytical procedure developed in this study provides a precise methodology that overcomes interference of endogenous akin compounds. It is less time consuming, and uses smaller amounts of organic solvents than published methods. Since the recovery of cholesterol is quantitative, the internal standard could be safely eliminated, and this features one of the most advantages of this assay. The methodology is rather straight forward and can be adopted for cholesterol determination in other products with slight modification.

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REFERENCES

Adams, M.L., D.M. Sullivan, R.L. Smith and E.F. Richter, 1986. Evaluation of direct saponification method for determination of cholesterol in meats. Assoc. Off. Anal. Chem. 69: 844-46.

Bair, C.W. and W.W. Marion, 1978. Yolk cholesterol in eggs from various avian species. Poultry Sci., 57: 1260-65.

ElDeeb, M.A., 1994. Effect of feeding garlic on quantitative and qualitative productivity of laying hens. Proceeding of The 8th Conference: The Egyptian Society for Animal Production (in press).

Emara, S., 1993. High-Performance Liquid Chromatographic Analysis of Certain Anthracyclines in Spiked Plasma and Urine. Ph.D. Dissertation, Assiut University, Assiut, Egypt.

Food and Nutrition Board, 1953. Publ. 75, Natl. Acad. Sci., Natl. Res., Council, Washington, D.C.

Hargis, P.S., 1988. Modifying egg yolk cholesterol in the domestic fowl- a review. World's Poultry Sci., 44: 17-29.

Maclean, N., 1987. Macmillan Dictionary of genetics and cell biology. The Macmillan Press Ltd., Great Britain.

Naber, E.C. and M.D. Biggert, 1985. Analysis for and generation of cholesterol oxidation products in egg yolk by heat treatment. Poultry Sci., 64: 341-47.

Sutton, C.D., W.M. Muir and Jr. G.E. Mitchell, 1984. Cholesterol metabolism in the laying hen as influenced by dietary cholesterol, caloric intake, and genotype. Poultry Sci., 63: 972-80.

Sloan, D.R., R.H. Harms, G.B. Russell and W.G. Smith, 1994. The relationship of egg cholesterol to serum cholesterol, serum calcium, feed consumption and dietary cholecalciferol. Poultry Sci., 73: 472-75.

Zlatkis, A., B. Zak and A.J. Boyle, 1953. A new method for the direct determination of serum cholesterol. J. Lab. Clin. Med. 41: 486-92.

التحليل الكرماتوجرافى للكوليسترول فى بيض الدجاج

مريم الديب[1] - سامى عمارة[2]

١- قسم الانتاج الحيوانى، كلية الزراعة، جامعة أسيوط، أسيوط ٢- قسم الكيمياء التحليلية الصيدلية - كلية الصيدلة جامعة أسيوط، أسيوط.

لقد تم إستخلاص الكولسيترول من صفار بيض الدجاج وتحليله باستخدام جهاز الكروماتوجرافيا السائلة تحت الضغط العالى (HPLC) المجهز بعمود تحليل مصنوع من الصلب الذى لا يصدأ بطول ١٥٠ مم ومقطع ٤,٦ مم ومعبأ يدويا من العينات بمادة السيلكا (ODS/TN) ذات المسام سعة ٨٠ انجستروم ولقد تم التأكد من الكولسيترول المستخلص من بيض التجارب بالمقارنة بعينة الكرولسترول المستورد من شركة سيجما ٠
واتضح ان هذه الطريقة المستحدثة - لتعيين الكولسيترول - على مستوى عال من الحساسية والدقة التى جعلت فى الامكان تقدير العديد من العينات المنفردة٠ وللتأكد من صدق النتائج اضيفت كمية معلوم من الكولسيترول الاصلية (من سيجما) الى صفار البيض ثم تم اجراء تجربة الاستخلاص والتقدير الكروماتوجرافى المستحدثة لعينة الصفار المضاف اليها الكولسيترول فكانت النتائج أكثر من ٩٩٪٠ ويحسن التنويه الى ان الطريقة المستحدثة مضبوطة (محكمة) ولا يحتاج مستقبليا الى معيار داخلى عند التحليل الكروماتوجرافى وبالاضافة الى ذلك فانه قد تأكد لدينا ان هذه الطريقة يمكن تطبيقها عند دراسة تأثير التغذية على كمية الكوليسترول فى بيض الدجاج٠