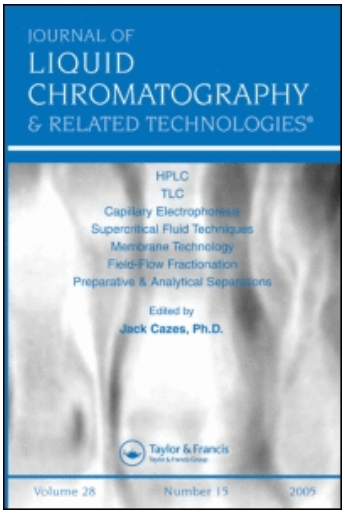

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DETERMINATION OF CHOLESTEROL IN EGG YOLK BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY USING AN AUTOMATED PRECOLUMN-SWITCHING PROCEDURE

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ABSTRACT

A direct injection high-performance liquid chromatographic method is developed for the determination of cholesterol in egg-yolk. The described method involved an on-line sample clean-up procedure using column-switching technique and protein-coated RP-18 precolumn. The egg-yolk was diluted with tris buffer, pH 8.5 to produce homogenous sample and render it suitable for injection. On the precolumn, cholesterol was preseparated from endogenous components of the egg-yolk with tris buffer, pH 8.5 for 4 minutes. To complete the precolumn washing cycle, the solvent selector is automatically changed from tris buffer to 0.05 M phosphate buffer, pH 4.8 for 2 min, followed by 15% methanol in 0.05 M phosphate buffer, pH 4.8 for 1 min further.

After column switching, the elute fraction containing cholesterol was further separated by reversed-phase chromatography using Spherisorb (ODS) column with a mobile phase consisting of acetonitrile, isopropanol, and phosphate buffer, pH 4 (40:50:10, v/v). The average cholesterol recoveries ranged from 97.70 to 100.50% and the relative standard deviations ranged from 2.70 to 3.84%. The method allows accurate and fast daily routine monitoring of cholesterol in large numbers of chicken eggs and is proven to be highly applicable in studying nutritional effects on chicken egg cholesterol.

INTRODUCTION

Cholesterol is a polycyclic ring lipid structure that has a multifunction role in the biological systems. 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-yolk, cheese, milk-butter, or lard as ingredients. It is now a common belief that a human diet low in saturated fats 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 Egyptian community has always considered the chicken egg as an important part of the diet. However, the recent international concern brings 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 a simple and rapid assay for cholesterol content to possibly study cholesterol 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.

Various analytical methods have been reported for the determination of cholesterol in tissues, foods, and body fluids. These include high performance liquid chromatography (HPLC),[1-3] thin layer chromatography (TLC),[4] gas chromatography (GC)[5,6] and spectrophotometry.[7,8] Although HPLC techniques are very sensitive, extraction procedures prior to analysis using considerable amounts of expensive and environmentally damaging organic solvents is a great drawback. In general, these techniques are tedious and less precise because it involves a series of liquid pipetting and dispensing steps. Gas chromatographic

methods can provide definite qualitative and quantitative results but require time-consuming derivatization steps. Obviously, there is always need for a reliable, reproducible, and simple assay of cholesterol in food products of animal origin.

In this manuscript, our study was involved in a research effort aimed at developing and improving a direct injection chromatographic technique using protein-coated RP-18 silica as a precolumn to isolate cholesterol from egg-yolk matrix prior to HPLC.

EXPERIMENTAL

Materials

Cholesterol (Sigma Chem. Co., St. Louis, MO, U.S.A.), Tris (hydroxymethyl)-methylamine, sodium chloride, potassium dihydrogen orthophosphate, disodium hydrogen phosphate, and sodium hydroxide (BDH Chemicals Ltd., Poole, U.K.) were used without further purification. Acetonitrile, 2-propanol, and dimethylformamide (DMF) (E. Merck AG, Darmstadt, Germany) were HPLC grade.

Eggs were obtained from commercial layers, single comb white Leghorn hens (Hy-Line), that were raised in the farm of the Department of Animal Production, Assiut University, Egypt.

Instruments

Automated on-line sample clean-up and enrichment were performed using a sequence programmer, Model SCY-PO (Omron, Tokyo, Japan) capable of controlling a solvent selector, Model 8V (Kyowa, Seimitsu, Tokyo, Japan) and a Model 7010 flow direction-switching valve (Rheodyne, Berkeley, California, U.S.A.). The automated column-switching system (Figure 1) is equipped with a Model 7125 sample injection valve (Rheodyne, Berkeley, California, U.S.A), and two isocratic pumps, Model CCPD for pump 1 and Model HLC 803D for pump 2 (Toyo, Soda, Japan).

This system was equipped with two columns: One was a short protein-coated RP-18 silica column, 20 µm (20 X 4.6 mm i.d.) for clean-up and enrichment of cholesterol from egg-yolk matrix. The other was an analytical column, Spherisorb ODS, 5 µm (250 X 4.6 mm i.d., µm) (Waters, Assoc., Milford, USA).

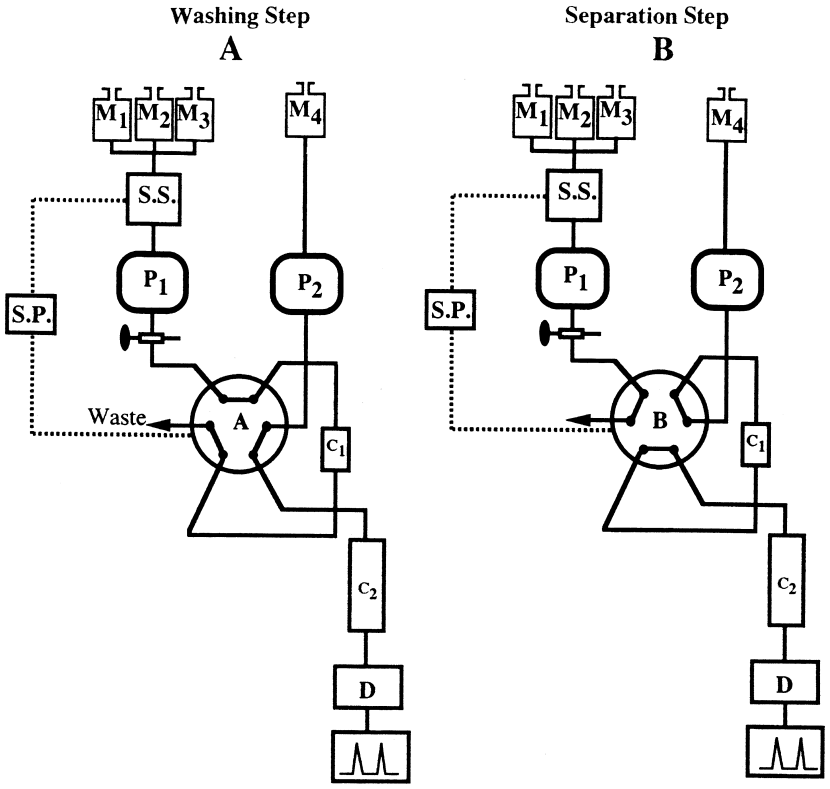

Figure 1. Schematic diagram of the HPLC apparatus for the analysis of cholesterol in egg-yolk. M_1, M_2, M_3 and M_4, mobile phases 1, 2, 3 and 4; P_1 and P_2, pumps 1 and 2; C_1, protein-coated precolumn; C_2, analytical column; S.S., solvent selector; S.P., sequence programmer; D, detector.

The protein-coated precolumn was prepared manually in our laboratory. The chromatographic detector was a spectrophotometric detector UV-8 Model II (Toyo Soda, Japan) operated at a wavelength of 212 nm. Peak heights were computed on a Shimadzu C-R3A chromatopac integrator (Shimadzu, Kyoto, Japan).

Cholesterol Standard Solutions

A stock solution of cholesterol (1.0 mg/mL) was prepared by dissolving an accurately weighed amount of 25 mg in 25 mL DMFA. Cholesterol working

standard solutions for calibration were prepared by serial dilution of appropriate volumes of the stock solution with DMFA to produce solutions in the concentration range 20-80 µg/mL.

Chromatographic Determination

An accurately weighed amount (0.5g) sample egg-yolk was quantitatively transferred to a 25-mL volumetric flask followed by 20 mL tris buffer, pH 8.5. The sample was mixed gently and completed to volume with the same solvent then thoroughly mixed. The resulting sample solution was pretreated in an ultrasonic bath to eliminate foaming. An aliquot of 250 µL was analyzed for cholesterol as follows:

The protein-coated precolumn into which the egg-yolk sample was injected, was equilibrated with tris buffer of pH 8.5 (**M1**) for 5 min. Then approximately 300-µL sample was drawn into a 0.5-mL syringe and a 25-mm, 0.45 µm filter was placed on a syringe tip. The syringe filter assembly was placed onto the Rheodyne injector, and the sample was loaded into the 250-µL sample loop. To drain out proteins and other hydrophilic compounds, the protein-coated precolumn was washed with **M1** for 4 min at a flow rate of 1.0 mL/min by pump 1. To complete the precolumn washing cycle, the solvent selector is automatically changed from **M1** to 0.05M phosphate buffer, pH 4.8 (**M2**) for 2 min followed by 15% methanol in 0.05M phosphate buffer, pH 4.8 (**M3**) for 1 min further. Then the switching valve was rotated to allow the analytical mobile phase (**M4**), of acetonitrile, isopropanol, and phosphate buffer, pH 4 (40:50:10, v/v), to flush the precolumn contents onto the analytical column (Spherisorb ODS). A period of 2 min later, the switching valve was rotated back to the original position to remove the rest of the organic solvent from the precolumn and to prepare the precolumn for another injection.

Parallel to this process, pump 2 delivers the analytical mobile phase (**M4**) to the analytical column, at a flow rate of 1mL/min, where cholesterol underwent chromatographic separation and quantitation. The analytical chromatography starts after 7 min (during this time, the precolumn is not connected to the analytical column). At the end of analysis, protein-coated precolumn was washed with distilled water for 30 min, then the whole system was washed with methanol for 15 min.

RESULTS AND DISCUSSION

A primary problem in the analysis for cholesterol in egg-yolk is the detection and quantitation of small amounts of this substance in the presence of relatively large amounts of endogenous components. Also, injection of egg-yolk

into conventional HPLC systems results in rapid degradation of performance typically evidenced by increased back pressure and reduced efficiency. These effects are usually attributed to protein denaturation at partitioning phase interface and accumulation in columns. The accumulation closes down the pores of silica particulates, inhibits analyte diffusional mass transport, and decreases column efficiency. After the first injection, the columns are rendered useless due to clogging of the interparticulate space. It has been reported that, protein-coated RP-18 silica had lost the adsorbability for proteins, but still had the characteristic of reversed phase packing materials for small hydrophobic molecules.[9] Accordingly, the use of column-switching techniques and protein-coated RP-18 precolumns enable us to separate cholesterol automatically from the endogenous components of egg-yolk matrix.

Sample Preparation

Before any procedure is adapted to chromatographic assay of cholesterol in chicken eggs, there is practical consideration to be made, such as whether the sample to be assayed needs pretreatment procedure. Egg-yolk was diluted with tris buffer (pH 8.5) to produce homogenous sample and render it suitable for injection. Also, filtration step was introduced while injection to assure analysis of egg-yolk cholesterol with no complication due to fluctuation in the precolumn pressure.

Chromatographic Separation

The extraction selectivity and resolution efficiency were optimized in preliminary experiments to minimize interference arising from endogenous components. This was achieved by optimizing pH, salt concentration, types and concentration of organic modifiers. Six random samples each of authentic and egg-yolk cholesterol was subjected to the assay procedure and the mean retention times were 8.43 and 8.41 min, respectively. The identity of the peak of cholesterol in egg-yolk was confirmed as follows: the coincidence of the retention time with that of the authentic cholesterol and the increase in peak height at the same retention time on addition of cholesterol to egg-yolk without the change in the peak shape. A typical chromatogram of cholesterol in egg-yolk is shown in Figure 2.

Optimization of Valve Timing for Column Switching

In the present work, the precolumn functions are to enrich the sample and clean-up cholesterol from endogenous constituents of egg-yolk matrix. Thus, the most critical interval is the first, where enough time must be given for the

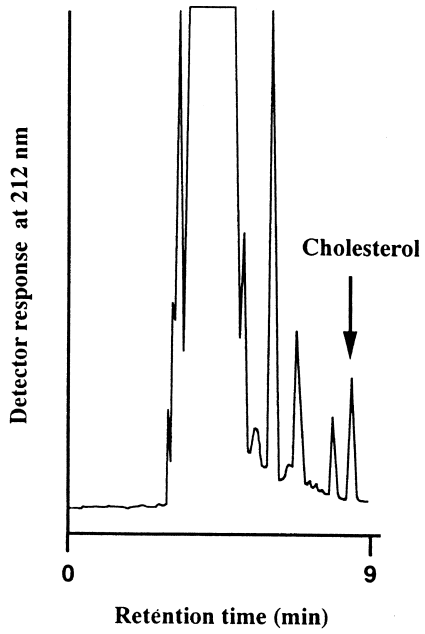

Figure 2. Typical chromatogram of cholesterol from egg-yolk sample.

interferences to elute off the precolumn while still retaining cholesterol. The washing-time needed careful investigation to keep the total run times as short as possible, and to exclude any transfer of egg-yolk proteins and other polar constituents to the analytical column, which would reduce the efficiency and the longevity of the analytical column. Clean-up of the trapped cholesterol with 4 mL of **M1** was not sufficiently effective to remove all the components that interferes with detection of cholesterol by means of UV monitoring. Therefore, second washing step using 2 mL of **M2** was introduced. Whereas the number of injections onto the protein-coated precolumn was high, an increase in the pressure on the analytical column was observed. This confirms the advantage of the additional washing step (**M3**) that provides low interference back ground signals and prevents a pressure increase on the analytical column.

The second interval (2min for **M4**) should allow complete elution of cholesterol from the precolumn after which the switching valve was rotated back to the injection mode (original position). While the analytical column is carrying out the desired separation, the precolumn is cleaned in preparation for the next sample.

Table 1

Recovery of Cholesterol Spiked in Egg Yolk

Concentration Spiked (μg/mL)	Recovery*(%, mean ±SD)		CV (%)
50	100.50	3.84	3.82
75	99.09	2.96	2.98
100	97.70	2.70	2.76

* Five replicate analyses of each concentration.

Relative Extraction Efficiency

The relative extraction efficiency of the extraction column was examined by comparing the peak height of authentic cholesterol when added to egg-yolk to those obtained by injection of a respective amount of cholesterol dissolved in DMFA under the same column-switching conditions.

The results presented in Table 1 illustrated that about 97.7-100.5% of cholesterol have been extracted and retained until elution. The enriched peak height was corrected by subtraction of the peak height corresponding to endogenous cholesterol in the egg-yolk sample.

As a result of the previous studies, it was clear that, egg-yolk proteins could not be adsorbed anymore on the external surface of protein-coated precolumn, and they could not enter the interior of small pores, thus they flowed out of the precolumn. But the small pores still retained the adsorptivity for cholesterol. The adsorbed cholesterol on the internal surface of small pores could be eluted by an appropriate eluent containing organic solvents (**M4**).

Precolumn Operating Technique

The analyte enriched on the precolumn was transferred to the anlytical column either in the straight flush mode (Figure 1) or in the back flush mode (Figure 3) and the recovery was dependent on the column coupling time. When the precolumn was back flushed, the recovery of cholesterol from pretreated egg-yolk reached values above 90% for column coupling time of 15 sec. In the straight flush mode the recovery was almost complete after 45 sec. The results indicated that cholesterol was adsorbed on the top of precolumn and was transferred faster in the back flush mode than in the straight mode.

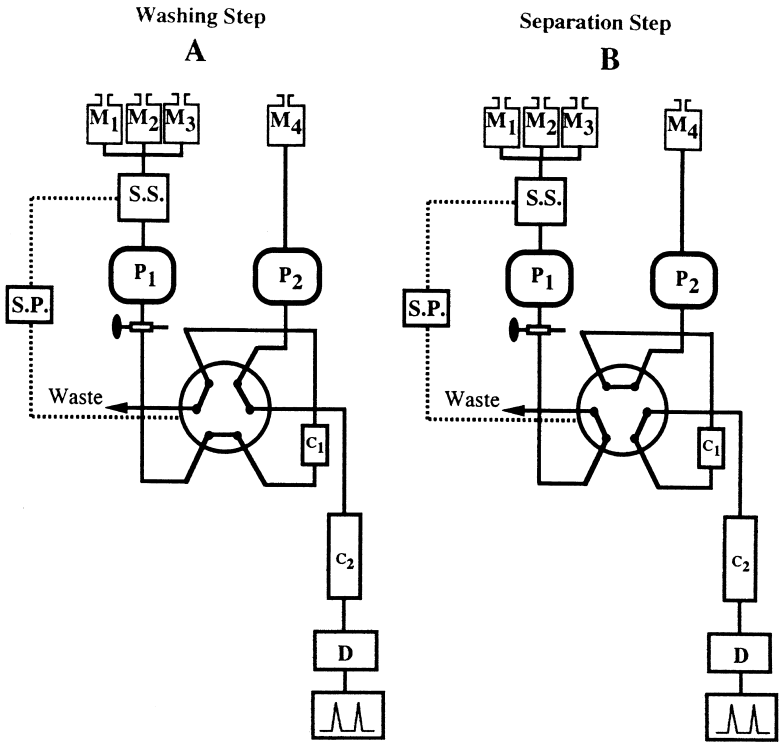

Figure 3. Block diagram of HPLC system for back flush mode.

A drawback to the back flush mode is that applying the mobile phase alternatively in opposite direction could disturb the precolumn. To ensure maximum recovery of cholesterol, the precolumn was operated for 2 min in the straight flush mode.

Quantification

A calibration curve was constructed by plotting the measured peak height of cholesterol versus concentration. A linear relationship between peak height (*Y*) and concentration (*X*) existed over the examined concentration range (20-80 µg/mL). The regression equation from which unknown samples were assayed for their cholesterol content was as follows:

$$Y = 0.2304 + 0.3020\ X \quad (r = 0.9993)$$

Table 2

Intra-day and Inter-day Accuracy and Precision of Cholesterol in Egg Yolk

	Concentration (μg/mL)			
	Added Conc.	Measured Conc. (Mean ±SD)	CV (%)	Mean RE (%)
Intra-assay*	20	39.86 3.96	3.97	-0.35
	40	60.04 3.02	3.01	0.06
	80	78.40 2.81	2.86	-2.00
Inter-assay**	20	39.34 4.43	4.50	-1.65
	40	59.37 3.85	3.89	-1.05
	80	77.44 3.69	3.70	-3.20

* Average of five determinations.

** Inter-day precision was determined from 6 different runs over 6 days.

Precision and accuracy of the analytical method were determined by analyzing three sets of egg-yolk samples containing known different concentrations of cholesterol. The coefficients of variance (CV) and the relative error (RE) of the mean measured concentrations were served as measures of accuracy and precision for validation of the assay procedure. The statistical data for the quantitation of cholesterol are presented in Table 2. The intra-day coefficients of variance and the mean relative errors ranged from 2.86 to 3.97% and from –2.00 to 0.06%, respectively, for the concentrations between 20 and 80 μg/L of cholesterol. The inter-day coefficients of variance and the mean relative errors ranged from 3.70 to 4.50% and from –3.20 to –1.05%, respectively, for the same concentration range.

Life Time of Protein-Coated Precolumn

In a series of about 125 analysis of cholesterol in egg-yolk samples, with an injection volume of 250 μL, where **M1**, **M2** and **M3** were used for washing the protein-coated precolumn, there was no need for a column exchange. The results showed constant quality as regards selectivity and resolution. A detectable increase in pressure of protein-coated precolumn was noted after about 150 injections. The protein-coated precolumn was, regardless of pressure, changed after 125 injections in order to keep contamination of analytical column down to the smallest amount possible. The preparation of protein-coated precolumn is simple and economic and reduces the time and cost of renewal.

Application

After recognization the identity of measured cholesterol in egg yolk (Figure 2), 15 eggs (from the same source) were individually assayed for their cholesterol content. It appears that cholesterol in chicken egg-yolk falls within a reasonable limit (about 360-400 mg/100g yolk). This distribution is consistent with previous findings[10] indicating the presence of sufficient genetic variability in yolk cholesterol level. Recent publication reported an overall average of egg-yolk cholesterol from Hy-line to be 364 mg/100g egg yolk. Our reported values are reasonable ranging within those of other published reports which confirms the overall precision and subsequent reproducibility of the developed technique. It is therefore, recommended that the described procedure is to be routinely used in poultry science laboratories for physiological and/or nutritional experiments.

CONCLUSIONS

The advantageous use of protein-coated RP-18 precolumn as a clean-up device for cholesterol from egg-yolk matrix in combination with a column switching-technique can utilize an on-line sample enrichment and clean-up procedure. It is less time consuming, and uses small amounts of organic solvents than other published methods. The possibility of error arising during the several pretreatment steps is considerably reduced. It provides a precise methodology that overcomes interference of endogenous akin components. Since the recovery of cholesterol is quantitative, the internal standard could be safely eliminated, and this feature was one of the most advantages of the assay.

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