AN IMPROVED METHOD OF CHOLESTEROL DETERMINATION IN EGG YOLK BY HPLC

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ABSTRACT

An improved method for cholesterol determination in egg yolk is reported in this paper. Egg yolk was first diluted. Cholesterol was then extracted with ether and petroleum ether, and quantified by reverse phase chromatography on a Zorbax ODS column (0.46 × 15cm, 5-6 µm) using a mobile phase of acetonitrile and 2-propanol (4:1) with a flow rate of 0.6 mL/min. A linear correlation was observed between cholesterol concentration at 0.05-0.40 mg/mL and its peak heights with a correlation coefficient of 0.9993 (n = 5). The average recovery was 98.9%, and detection limit was 0.02 mg/mL. No differences in cholesterol concentration were observed between egg yolk samples with and without saponification. Rapid and reproducible quantification of cholesterol in egg yolk can be completed with this simplified method.

INTRODUCTION

Elucidation of the physiological role of cholesterol esters in the genesis of atherosclerosis has resulted in great interest in the cholesterol distribution among different foods, and the factors affecting the cholesterol concentration. This has led to numerous attempts to develop rapid, accurate and convenient methods for determination of cholesterol.

Eggs are highly favored in the food industry as an excellent source of some nutrients and for their valuable functional properties (Cook and Briggs 1977). High cholesterol content in yolk has, however, caused great concern. The average values reported by the USDA Marketing Service are 213 mg per large

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egg and 12.5 mg/g of yolk, assuming a weight of 17 g for a large egg (USDA 1989). Egg yolk cholesterol values varying from 9.21 to 22.8 mg/g of yolk have been reported (Cunningham et al. 1974; Nix et al. 1974; Washburn and Nix 1974; Bar and Marion 1978; Ingr and Simeonova 1983). Enormous efforts have been made to reduce cholesterol levels; thus, requiring a simple, rapid and reproducible method for cholesterol determination.

Various kinds of methods are available for cholesterol determination in foods, including spectrophotometry (Abell et al. 1952; Folch et al. 1957; Bachman et al. 1976; Bohac et al. 1988), gas-liquid chromatography (Punwar 1975; Beyer et al. 1989; Tsui 1989), high performance liquid chromatography (Newkirk and Sheppard 1981; Richard and Larry 1981; Hurst et al. 1984; Kou and Holmes 1985; Indyk 1990; Bocos et al. 1992; Casiraghi et al. 1994) and others (Allain et al. 1974; Papastathopoulos and Rechnitz 1975; Shen et al. 1982; Contreras et al. 1992; Crockett and Hazel 1995). Due to the water-insoluble nature of cholesterol, its determination is always complicated by the coexisting lipids. Consequently, all reported procedures inevitably included a saponification-extraction step, and a multistage solvent extraction followed by purification and concentration. Sample saponification, in particular, is the major cause of operational inconvenience and complications.

The objective of the present study was to develop an assay method for cholesterol determination in chicken egg yolk by high performance chromatography without a requirement for sample pretreatment with saponification.

MATERIALS AND METHODS

Preparation of Egg Yolk Samples for Cholesterol Determination

Fresh chicken eggs were purchased from a local market. Twelve eggs were randomly divided into three groups, samples A, B, C. Egg yolk was separated from the white, and washed with distilled water and then rolled on filter paper to remove as much of the adhering egg white as possible. The yolk membrane was then punctured, and 4 yolks in each group were pooled and thoroughly mixed. Well mixed egg yolk was sampled for analysis throughout this work.

Egg Yolk Samples Without Saponification. Egg yolk samples of approximately 2 g from each group were accurately weighed and diluted to 20 mL with distilled water, then mixed completely. Diluted egg yolk (1.0 mL) was pipetted into a 15-mL test tube with a stopper; and 1.0 mL of 95% ethanol was added into the same test tube and mixed well to facilitate the subsequent extraction. The mixture was first mixed with 2.5 mL of ether, and then adequately mixed with 2.5 mL of petroleum ether (b.p. 30-60C). After standing
for 30 min at ambient temperature. 0.5 mL of the organic phase was pipetted into an Eppendorf tube (1.5 mL) and evaporated to dryness under a stream of nitrogen (45°C). The residue was dissolved in 0.5 mL of ethanol, thus constituting the egg yolk samples without saponification, and 10 μL of the solution was applied to the HPLC system for cholesterol determination.

**Egg Yolk Samples With Saponification.** Saponified egg yolk samples were also prepared. Diluted egg yolk (1.0 mL) was pipetted into a test tube with a stopper (25 mL), 3.0 mL of 95% ethanol was added to the sample and the test tube was vigorously shaken to facilitate the subsequent treatment. Saponification of the sample was carried out according to Sim and Bragg (1977) by adding 2.0 mL of 50% potassium hydroxide solution to the test tube, and the loosely capped tube was incubated for 60 min at 50°C. During incubation, periodic agitation was applied to ensure efficient digestion of lipid. After cooling to ambient temperature in an ice-water bath, extraction solvent (5.0 mL of hexane) was added, and the tubes were shaken for 5 min after being stoppered securely. Distilled water (3.0 mL) was added to the above mixture, then the tube was capped and shaken ten times. The resulting mixture was centrifuged at 1000 × g for 10 min to produce a satisfactory phase separation. The organic phase (0.5 mL) was pipetted into an Eppendorf tube (1.5 mL) and evaporated to dryness under a stream of nitrogen (45°C). The residue was dissolved in 0.5 mL of ethanol and constituting the egg yolk samples with saponification. A 10 μL aliquot of the solution was applied to the HPLC system for cholesterol determination.

**HPLC Determination of Cholesterol**

**HPLC Analysis.** HPLC analysis was carried out at ambient temperature with a TSK Model CCPD high-speed liquid chromatograph (Tosoh Co. Ltd., Toyo, Japan) on a Zorbax ODS column (0.45 × 15 cm) (Dupont Instruments, Wilmington, DE). The eluate was monitored by a TSK Model UV-8000 detector (Tosoh Co. Ltd., Toyo, Japan) at 208 nm. The mobile phase was an isocratic mixture of acetonitrile and 2-propanol (4:1) and the flow rate was 0.60 mL/min. Sample injection volumes were 10 μL. All the chromatographic data were obtained by averaging at least 5 replicates.

**Cholesterol Standards.** Cholesterol was purchased from Sigma (St. Louis, MO). All solvents used were HPLC grade or analytical grade purchased from Biological Science and Technology of Shanghai Co. Ltd. (Shanghai, China).

Cholesterol was dissolved in ethanol (1 mg/mL) as stock standard solution and stored at -20°C. A series of working standards was obtained by diluting the stock standard solution in the same medium to obtain concentrations ranging from 0 to 0.40 mg cholesterol/mL. A 10 μL aliquot of the working solution was
applied to the HPLC system. Correlation between cholesterol concentration and its chromatographic peak height was established.

**Identification of Cholesterol.** The cholesterol peak fractions from HPLC of egg yolk samples with and without saponification were collected. Its purity was determined by spectrophotometric scanning using an UV/VIS Spectrometer Lambda Bio10 (Perkin Elmer Co. Ltd.).

**Estimation of Cholesterol Recovery from Egg Yolk Samples Without Saponification.** Approximately 2.0 g of egg yolk sample was accurately weighed and diluted to 20 mL with distilled water, then mixed completely. Diluted egg yolk (1.0 mL) was pipetted into a 15-mL test tube with a stopper, then increasing amounts of cholesterol (0.075, 0.200, 0.325 mg) were added, and treated as described for samples without saponification. The final solution (10 μL) was applied to the HPLC system. Recovery of cholesterol from egg yolk samples without saponification was estimated by comparing the externally added cholesterol content in the samples as determined by this method with the externally added amounts of cholesterol in the samples.

**Cholesterol Determination by Colorimetry**

Cholesterol content of the saponified egg yolk samples was also determined by colorimetry according to the methods of Abell *et al.* (1952) and Folch *et al.* (1957). Egg yolk samples were subjected to alcoholic KOH saponification and hexane extraction prior to color reaction with Liebermann-Burchard reagent according to the same procedures of saponification as in the sample preparation for HPLC analysis. A 1-mL portion of the organic phase was pipetted into a 15-mL test tube with a stopper and evaporated to dryness under a stream of nitrogen (45°C). Then 6.0 mL of Liebermann-Burchard reagent was added to the same test tube, and adequately vortexed. Absorbance was measured at 640 nm after standing for 30 min at ambient temperature, using a UV/VIS Spectrometer Lambda Bio10 (Perkin Elmer Co. Ltd.). Blank and standard solutions were prepared by exactly the same procedure as for the egg yolk samples.

**Statistical Analysis**

A one-way ANOVA was used to analyze the effect of cholesterol assay methods on egg cholesterol values. Assay methods were the main effects, with the mean square of method by replication as the error term. Duncan's multiple range test (Steel and Torrie 1980) was used to differentiate treatment means.
RESULTS AND DISCUSSION

Calibration of Cholesterol Standard

A reverse phase chromatogram of a cholesterol standard is shown in Fig. 1. The retention time for cholesterol was 19 min. A satisfactory linear correlation between cholesterol concentration and its peak height was obtained as is shown in Fig. 2. The regression equation was calculated to be \( Y = 44.845X - 0.071 \) with a correlation coefficient of 0.9993 (\( n = 5 \)) within the cholesterol concentration range from 0 to 0.40 mg/mL. Perfect linearity in the cholesterol concentration range indicated no interaction between cholesterol and the column matrix.

\[ \text{FIG. 1. HPLC SEPARATION OF STANDARD CHOLESTEROL} \]

Conditions: Column, Zorbax ODS (0.46 × 15 cm, 5–6 μm); Mobile phase, acetonitrile: 2-propanol (V:V = 4:1); Flow rate, 0.6 mL/min; Detector, UV-208 nm; Injection volume, 10 μL.

Determination of Cholesterol Content in Egg Yolk

Chromatograms of egg yolk samples with and without saponification are shown in Fig. 3B and 3A, respectively. In both chromatograms, cholesterol of the yolk samples was eluted at the same retention time, 19 min, in good accord
R.-Z. ZHANG, L.L., S.-T. LIU, R.-M. CHEN and P.-F. RAO

Concentration of cholesterol (mg/mL)

FIG. 2. THE CALIBRATION GRAPH OF CHOLESTEROL

with result of the cholesterol standards. The UV/Visible spectra of the collected cholesterol peaks from yolk samples with or without saponification were exactly the same as that of the cholesterol standard, indicating the successful isolation of cholesterol by the selected HPLC system. Small peaks can be observed in the chromatograms of the egg yolk sample without saponification, but these were almost completely absent in the chromatograms of the saponified sample. As is shown in Fig. 3A, egg yolk lipids were completely separated from the cholesterol; therefore, there was no interference due to egg lipids with cholesterol quantification in the yolk sample without saponification. As a matter of fact, cholesterol peaks on both chromatograms are of similar heights, indicating no serious discrepancy between egg yolk samples with and without saponification. It is apparent that with the set of chromatographic conditions adopted in this study, reliable results of cholesterol determination could be obtained without the complicated sample treatment of saponification.

Listed in Table 1 are results of cholesterol determination by colorimetry and HPLC (5 replicates) of the egg yolk samples prepared by different treatments. Cholesterol content is expressed as milligrams per gram of yolk. As shown in Table 1, cholesterol values of chicken eggs, as determined by both colorimetry and HPLC methods, agreed with the reported values (9.21 to 22.8 mg/g of yolk), implying that HPLC determination of cholesterol in the yolk sample without saponification may be a useful alternative method for cholesterol determination in egg yolk. While the former can only be applied to the saponified egg sample, the latter can save great deal of labor by omitting saponification.
DETERMINATION OF CHOLESTEROL IN EGG YOLK

FIG. 3A. HPLC SEPARATION OF SAMPLE WITHOUT SAPONIFICATION
Conditions: Column, Zorbax ODS (0.46 × 15cm, 5-6 μm); Mobile phase, acetonitrile: 2-propanol (V:V=4:1); Flow rate, 0.6mL/min; Detector, UV-208 nm; Injection volume, 10 μL.

FIG. 3B. HPLC SEPARATION OF SAMPLE WITH SAPONIFICATION
Conditions: Column, Zorbax ODS (0.46 × 15cm, 5-6 μm); Mobile phase, acetonitrile: 2-propanol (V:V=4:1); Flow rate, 0.6mL/min; Detector, UV-208 nm; Injection volume, 10 μL.
TABLE 1.
COMPARISON OF EGG CHOLESTEROL DETERMINATION BY COLORIMETRIC AND
HPLC METHODS

<table>
<thead>
<tr>
<th>Yolk Samples</th>
<th>Colorimetry of Samples with Saponification</th>
<th>Samples with Saponification</th>
<th>Samples Without Saponification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/g yolk CV%</td>
<td>mg/g yolk CV%</td>
<td>mg/g yolk CV%</td>
</tr>
<tr>
<td>A</td>
<td>16.38± 0.14 0.85</td>
<td>15.30± 0.18 1.18</td>
<td>15.37± 0.18 1.17</td>
</tr>
<tr>
<td>B</td>
<td>12.10± 0.21 1.74</td>
<td>10.72± 0.15 1.40</td>
<td>10.77± 0.09 0.84</td>
</tr>
<tr>
<td>C</td>
<td>11.04± 0.17 1.54</td>
<td>9.46± 0.14 1.48</td>
<td>9.65± 0.16 1.66</td>
</tr>
</tbody>
</table>

1 Average (SD) egg weight: A, 55.40 ± 2.07g; B, 60.55 ± 2.65g; C, 61.08 ± 1.79 g.
2 Average (SD) yolk weight: A, 13.70 ± 0.84g; B, 13.97 ± 0.65g; C, 15.54 ± 0.40g.

Means within the same row with no common superscripts are significantly different (P < 0.01).

As is clear from Table 1, egg yolk cholesterol values obtained by colorimetry were significantly higher (P < 0.01) than those determined by the HPLC method (5 replicates). This discrepancy can be attributed to interference by coexisting lipids such as triglycerides, or free fatty acids, which can also be extracted into organic solvents and thus interfere with the color formation in the cholesterol colorimetry assay (Bohac et al. 1988). This assumption is well supported by our results shown in Fig. 3B. While most fractions other than cholesterol in Fig. 3A disappear as the result of saponification, some still remained even after exhaustive saponification for 60 min. Overestimation occurred as the result of interference of those fractions in colorimetry. Accordingly, cholesterol determination of egg yolk by HPLC with or without saponification is more reliable than colorimetric determination with saponification.

As shown in Table 1, accuracy of cholesterol determination by HPLC without saponification (relative standard deviation, RSD) obtained for 5 replicates on the same sample (sample A) was 1.17%. RSD for sample B and for sample C was 0.84% and 1.66%, respectively. Comparison of results from HPLC determination with saponification and the colorimetric method, showed that a similar accuracy was obtained for cholesterol determination of egg yolk by HPLC without saponification.
As is shown in Table 2, the values determined for the externally added amounts of cholesterol in the egg yolk samples without saponification were in good agreement with the actual values, indicating that omission of the saponification treatment of egg yolk samples caused no effect on cholesterol recovery in HPLC determination. This provides further evidence of the feasibility of HPLC determination of cholesterol in egg yolk samples without saponification.

<table>
<thead>
<tr>
<th>Cholesterol Added (mg)</th>
<th>Cholesterol Recovered (mg)</th>
<th>Recovery (%)</th>
<th>Average Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.075</td>
<td>0.074</td>
<td>98.7</td>
<td></td>
</tr>
<tr>
<td>0.200</td>
<td>0.198</td>
<td>99.0</td>
<td>98.9</td>
</tr>
<tr>
<td>0.325</td>
<td>0.322</td>
<td>99.1</td>
<td></td>
</tr>
</tbody>
</table>

n = replicates. ¹ Base level of 12.40 mg cholesterol / g yolk deducted.

From Table 1 and Table 2, it is clear that excellent accuracy and reproducibility can be expected for HPLC determination of cholesterol of the yolk samples with and without saponification, and satisfactory recovery of cholesterol was achieved in the proposed method of HPLC determination of the egg yolk samples without saponification. Although cholesterol values of yolk samples without saponification were slightly higher than those of the saponified yolk samples, the differences were nonsignificant, as is indicated in Table 1.

CONCLUSION

Superior to the colorimetric method and comparable to cholesterol determination of egg yolk samples with saponification by HPLC, this proposed method of HPLC determination without saponification can be used to determine cholesterol content in egg yolk in a much more efficient and convenient manner.

ACKNOWLEDGMENTS

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REFERENCES


DETERMINATION OF CHOLESTEROL IN EGG YOLK


