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Determination of egg yolk cholesterol content by on-column capillary gas chromatography

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ABSTRACT

A rapid and accurate method for cholesterol determination by on-column capillary gas chromatography was developed and evaluated with three different yolk preparations. The cholesterol content of fresh, frozen and dried egg yolk was determined by either direct saponification or by chloroform–methanol (2:1, v/v) extraction prior to saponification. 5-α-Cholestane was used as an internal standard. The effect of sample size, quantity of triglycerides (soybean oil) and efficiency of hexane extraction were evaluated.

The simple procedure for sample preparation eliminates the need for lipid extraction prior to saponification and multiple hexane extractions after saponification. Excellent precision and linearity were achieved.

INTRODUCTION

Accurate determination of cholesterol is of great importance to the food industry and has been of special interest to poultry scientists. A recent report [1] has emphasized some of the problems in quantitating cholesterol in foods. Lack of standardization of methods can lead to a large discrepancy between cholesterol values reported. Some authors [1–4] have discussed the problems and limitations of colorimetric methods which report values higher than those produced with gas chromatography (GC). A number of GC methods have been published for determination of cholesterol in various types of samples: serum [2], meats [4,5], milk [6] and eggs [3,7]. Most of these reported procedures used either a packed column or a capillary column with a split-injection technique, and differ in sample preparation, often involving time-consuming steps such as lipid extraction, refluxing with large volumes of solvents, filtration, clean-up by solid-phase extraction [6], and derivatization of cholesterol to its trimethylsilyl ether before GC analysis [8]. Kovacs *et al.* [9] have pointed out some of the problems associated with derivatization of sterols prior to GC analysis.

In this paper a GC method for determination of cholesterol content of fresh, frozen and dried egg yolk is developed. It involves a one-step sample preparation and a direct on-column injection for simple and accurate quantification of cholesterol. The method has been evaluated for its precision and accuracy. Factors which may influence the cholesterol determination that were examined are: direct saponification *versus*

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chloroform–methanol (2:1, v/v) extraction before saponification; sample size; effect of triglyceride levels in samples and the efficiency of hexane extraction after saponification.

MATERIALS AND METHODS

Sample preparation

Thirty eggs were divided into three groups of ten eggs each. Egg yolks from two groups were carefully and completely separated from the albumen, pooled and mixed thoroughly within each group. One group (fresh) was analyzed for cholesterol content immediately. The other group (frozen) was kept frozen at −20°C until analyzed. The third group of eggs (dried) were hard boiled, yolks were separated, pooled, mixed thoroughly and air-dried overnight in a fumehood. The dry-matter content of the dried sample was determined by taking subsamples in triplicate and drying at 110°C overnight in a forced air oven.

Direct versus post-extraction saponification

To test the need of lipid extraction prior to saponification, ten replicates from each of the three yolk preparations were saponified directly or after lipid extraction with chloroform–methanol (2:1, v/v) according to the method of Folch *et al.* [10] with minor modification. A weighed amount (0.2 g) of yolk sample was placed into a 50 × 25 mm PTFE-lined screw-cap tube, and 1 ml of the internal standard (IS) solution containing 2 mg of 5-α-cholestane (Sigma, St. Louis, MO, U.S.A.) in hexane was added to each sample. For lipid extraction, samples were homogenized with 20 ml of chloroform–methanol (2:1, v/v), using a Polytron homogenizer (Brinkmann Instruments, Westberg, NY, U.S.A.). After washing the Polytron head with approximately 2 ml of chloroform–methanol (2:1) solution, contents were transferred to a graduated cylinder and allowed to stand for 2 h. Deionized water was then added at 20% of total volume, cylinders were shaken and left for phases to separate. A 10-ml aliquot of chloroform layer was taken and solvent evaporated under nitrogen at 60°C in a heating block.

For saponification, 10 ml of alcoholic KOH solution (9.4 ml 95% ethanol plus 0.6 ml 33% KOH) were added to lipid extracts or weighed yolk samples. Tubes were capped tightly, mixed and heated at 60°C in a water bath for 1 h with occasional shaking. After cooling to room temperature, 5 ml of deionized water and 10 ml of hexane were added, and cholesterol extracted by vigorously shaking the tubes for 1 min. After separation of the layers, the upper hexane layer was diluted to a concentration of 1 mg/ml and analyzed directly by GC.

Efficiency of hexane extraction

To examine the efficiency of hexane extraction, a second extraction with 10 ml hexane and a known amount of internal standard was performed on the lower aqueous phase after it was carefully freed of all traces of the first hexane extract.

Linearity study

Aliquotes of a cholesterol (CH) standard (2 mg/ml of hexane) were taken to give a range from 1.2 to 6.0 mg CH per tube. Standards were treated in the same way as the

samples, including the addition of internal standard, saponification and extraction. Four standards with a weight ratio of 1.0 (CH/IS) were extracted with chloroform–methanol prior to saponification, and another four standards with weight ratio of 0.8 (CH/IS) were analyzed directly without saponification.

Effect of triglyceride levels

To evaluate the effects of triglyceride (TG) levels in the saponification mixture on the extraction of cholesterol, 0–170 mg of soybean oil was added to standard solutions containing 2 mg IS and 1.6 mg CH, saponified and extracted with hexane as described above.

GC operation

A Varian 3400 gas chromatograph equipped with an on-column injector, an 8035 autosampler and a flame ionization detector was used. A 30 m × 0.25 mm I.D. fused-silica capillary column SE-30 (Supelco, Bellefonte, PA, U.S.A.) with 0.25 μm film thickness was used under following conditions: oven temperature was held at 70°C for 0.1 min, then programmed at 40°C/min to 300°C and held there for 5 min. Injection temperature was programmed from 80 to 300°C at 150°C/min and held at 300°C for 7 min. Liquid CO_2 was used to cool the injector. Injection volume was 0.5 μl at a fast injection rate. Helium carrier-gas head pressure was set at 20 p.s.i. (*ca.* 1.5 ml/min) and a flow-rate of 30 ml/min as a make-up gas. Hydrogen and air gas flow to the detector were 30 and 300 ml/min, respectively. Detector temperature was at 300°C. A Hewlett-Packard Series 3353 laboratory automation system (Avondale, PA, U.S.A.) was used to integrate peak areas.

Calculation and statistical analysis

The relative response factor (RF) was calculated as a ratio of IS peak area to CH peak area multiplied by the ratio of the amount of CH to that of IS in standards. That is, RF = (area IS/area CH) × (amount of CH/amount of IS).

The cholesterol concentrations of yolk samples were calculated as follows: cholesterol content (mg/g of fresh yolk) = [(mg IS × area CH)/(area IS × g sample)]RF.

For the dried preparation, the weight of the sample was recalculated on fresh yolk basis for easy comparison between fresh, frozen and dried samples.

Analysis of variance (ANOVA) was applied to data pertaining to the extraction/saponification study (2 saponification procedures × 3 yolk preparations) and sample-size study. Orthogonal polynomials were used to examine the effect of various levels of TG in the saponification mixture and the effect of yolk sample-size on the efficiency of cholesterol extraction by hexane [11].

RESULTS AND DISCUSSION

A plot of the ratios of peak area of CH to IS *versus* their weight ratios (Fig. 1) gave a linear response in the range 1.2–6.0 mg cholesterol per tube. RF values obtained from standards carried through chloroform–methanol extraction, 1.087 ± 0.012 (mean ± S.E.M., $n = 4$), and direct saponification, 1.089 ± 0.023 ($n = 10$), did not differ from standards which were not saponified (1.056 ± 0.014, $n = 4$), indicating no loss of either cholesterol or internal standard during the entire procedure.

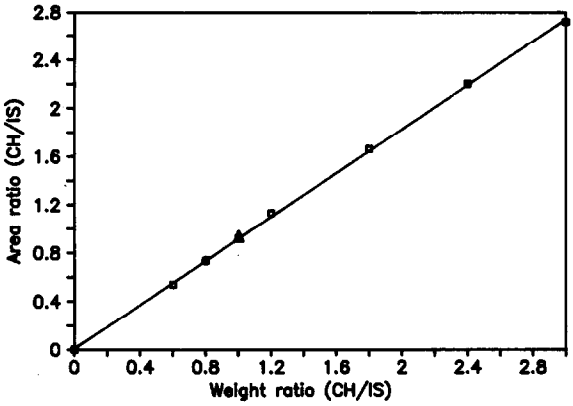

Fig. 1. The plot of area ratios *versus* weight ratios of cholesterol/5-α-cholestane demonstrating the linearity of response of cholesterol in the range 1.2–6.0 mg. Standards were subjected to either direct saponification (□, $n = 2$), Folch extraction prior to saponification (●, $n = 4$) or no saponification (△, $n = 2$).

Analyzing underivatized cholesterol on packed columns [9,12] leads to adsorption of cholesterol on the coated solid support and shorter column life. These authors [9,12] found it necessary to inject high concentrations of cholesterol every day to resaturate the absorption sites and restore stable response factors. Capillary columns minimize this problem. However, a split-injection technique can still lead to poor reproducibility and quantification of cholesterol by GC. With a cold on-column injection technique, the problem of adsorption is almost eliminated, and slight broadening of the cholseterol peak does not change RF values. As many as 1000 injections can be made before a short length of the column is broken off to restore the peak shape. A chromatogram of egg yolk cholesterol and internal standard after direct saponification is shown in Fig. 2. Excellent separation without any interfering peaks

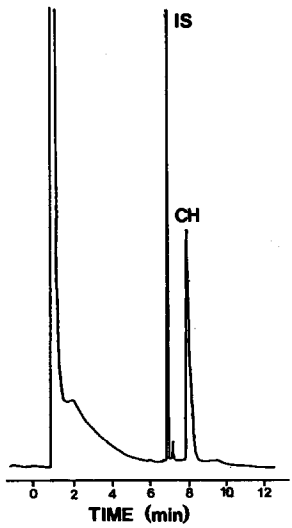

Fig. 2. Gas chromatogram of cholesterol and 5-α-cholestane of an egg yolk sample prepared by direct saponification as described in the text.

TABLE I

CHOLESTEROL CONTENT OF FRESH, FROZEN AND DRIED EGG YOLK BY GC ANALYSIS WITH EITHER DIRECT SAPONIFICATION OR FOLCH EXTRACTION PRIOR TO SAPONIFICATION

Preparations	Cholesterol content (mg/g fresh yolk) (mean ± S.E.M., $n = 10$)	
	Direct saponification	Folch extraction
Fresh	11.874 ± 0.050	11.892 ± 0.049
Frozen	11.808 ± 0.049	11.738 ± 0.045
Dried	11.589 ± 0.074	11.822 ± 0.070

was obtained. With a total analysis time of 20 min as many as 50–60 samples can be analyzed in one day with an autosampler.

Methods based on direct saponification, and chloroform–methanol extraction prior to saponification were not significantly different between fresh, frozen and dried preparations (Table I). Thus, lipid extraction before saponification is not needed for yolk cholesterol determination by the GC method described in this paper. Lillienberg and Svanborg [2] also found no difference between the two methods for plasma cholesterol determination.

Slover *et al.* [8] reported that the presence of triglycerides in the saponification mixture can affect the extraction of sterols. When increased levels of soybean oil were added to cholesterol reference standards before saponification, RF values of the first extraction were significantly raised ($p < 0.01$) (Table II). The percentage recovery of cholesterol by the first extraction was also affected ($p < 0.001$), resulting in lower efficiency of cholesterol extraction (Table II). When orthogonal polynomials are applied, the effects of TG levels on the efficiency and RF values are significant in both linear ($p < 0.001$) and quadratic terms ($p < 0.05$). Therefore, the amount of TG in the sample should be *ca.* $\leqslant 50$ mg for a more quantitative recovery of cholesterol from the saponification mixture.

TABLE II

EFFECT OF ADDING SOYBEAN OIL TO CHOLESTEROL STANDARDS ON CHOLESTEROL EXTRACTION BY HEXANE

Soybean oil added (mg/standard)	First extraction as % of total[a]	Response factors of first extraction[a]
0	98.204 ± 0.063	1.084 ± 0.002
40	96.847 ± 0.172	1.109 ± 0.009
80	95.789 ± 0.178	1.154 ± 0.006
130	94.441 ± 0.120	1.177 ± 0.003
170	94.053 ± 0.073	1.177 ± 0.003
Significance	$p < 0.0001$	$p < 0.001$
Linear term	$p < 0.0001$	$p < 0.0001$
Quadratic term	$p < 0.05$	$p < 0.05$

[a] Mean ± S.E.M., $n = 2$.

TABLE III

EFFECT OF SAMPLE SIZE ON CHOLESTEROL CONTENT OF THREE YOLK PREPARATIONS DETERMINED BY GC

Sample weight (g, fresh yolk equivalent)	Cholesterol (mg/g fresh yolk) (mean ± S.E.M., $n = 2$)		
	Fresh	Frozen	Dried
0.10	12.233 ± 0.114	11.920 ± 0.120	11.340 ± 0.460
0.20	11.594 ± 0.145	11.985 ± 0.095	11.220 ± 0.270
0.30	11.800 ± 0.049	12.000 ± 0.030	11.450 ± 0.050
0.40	11.667 ± 0.078	11.870 ± 0.020	11.660 ± 0.280
0.50	11.576 ± 0.060	11.610 ± 0.040	11.565 ± 0.345
Significance	NS[a]	NS	NS

[a] NS = Not significant.

The effect of yolk sample-size on linearity and cholesterol content was evaluated (Table III). For the three yolk preparations, the determined cholesterol contents were not significantly influenced by sample-size in the range 0.10–0.50 g fresh yolk equivalent.

To keep the procedure as simple as possible, the need for multiple extractions [9,13] with hexane after saponification was evaluated. The results of efficiency of cholesterol extraction after direct saponification with a 10-ml aliquot of hexane are shown in Table IV for dried yolk samples and standards with increasing amounts of cholesterol. The results show that one extraction removed >98% of cholesterol from standards, and there was no difference due to increasing amounts of cholesterol. With a sample-size ⩽0.2 g fresh yolk, the same percentage recovery (>98%) was achieved, showing quantitative extraction of cholesterol with the first 10 ml of hexane; therefore, the second extraction would not be necessary. As yolk sample size increased to 0.50 g,

TABLE IV

EFFICIENCY OF CHOLESTEROL EXTRACTION WITH HEXANE AFTER DIRECT SAPONIFICATION OF DRIED YOLK SAMPLES AND STANDARDS

Fresh yolk equivalent		Cholesterol standards	
Yolk (g)	First extract as % of total[a]	Cholesterol (mg/standard)	First extract as % of total[a]
0.10	98.079 ± 0.140	1.2	98.447 ± 0.117
0.20	98.189 ± 0.076	2.4	98.648 ± 0.028
0.30	97.455 ± 0.127	3.6	98.578 ± 0.021
0.40	96.618 ± 0.055	4.8	98.635 ± 0.114
0.50	96.661 ± 0.016	6.0	98.444 ± 0.137
Significance	$p < 0.0001$		NS[b]
Linear	$p < 0.0001$		NS

[a] Mean ± S.E.M., $n = 2$.
[b] NS = Not significant.

the percentage recovery by the first extraction was reduced to 96.6% which was significantly different at $p < 0.05$ level. The slight decrease of percentage recovery could be due to the increasing amount of lipid as the sample weight increases. An amount of 0.5 g of fresh egg yolk would provide about 150 mg of TGs, which at this level was shown to reduce the percentage recovery of cholesterol from standards (Table II).

Precision of the GC analysis step alone and the entire procedure was tested by ten injections of the same hexane extract of one sample and ten replicates of the same egg yolk carried through the entire procedure. The mean and the S.E.M. was 10.111 ± 0.028 mg/g yolk for GC alone and 11.808 ± 0.049 for the entire procedure, demonstrating excellent precision of the method.

The method reported here is a greatly simplified GC procedure for determination of cholesterol in egg yolk. The one-step sample preparation involving direct saponification of $\leqslant 0.20$ g fresh yolk followed by single extraction with hexane and direct analysis on GC offers a rapid method with excellent precision and accuracy.

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