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Rapid determination of total cholesterol in egg yolk using commercial diagnostic cholesterol reagent

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A rapid and accurate method for cholesterol determination using a commercial diagnostic cholesterol reagent ('enzyme method') was developed and evaluated with four different yolk preparations. The cholesterol content of The National Institute of Standards and Technology (NIST) Standard Reference Material in whole egg powder, fresh, frozen, and dried egg yolk was determined using gas chromatography and the enzyme method. All samples were subjected to direct saponification and solubilization prior to analysis. No treatment was applied to control samples, which were analyzed by the enzyme method. Solubilization of samples was performed at 0.85, 2 and 5% (w/v) NaCl concentrations. Solubilization measured in terms of cholesterol value was best at 2 and 5% NaCl levels. For egg powder standard and fresh or frozen egg, the cholesterol values obtained by the enzyme method from saponified or solubilized samples were not significantly different from values obtained by gas chromatography. For solubilized dried egg yolk the enzyme method gave results indistinguishable from chromatography for saponified yolk. However, the results obtained by the enzyme method and gas chromatography from saponified dried egg yolk were significantly different. The simple and rapid procedure developed for sample preparation (solubilization) eliminates the need for saponification prior to cholesterol determination using the enzyme method, and provides an alternative to an expensive and time-consuming gas chromatographic method. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

An accurate and rapid determination of egg cholesterol is of great importance to the food industry and especially to scientists working on several aspects of egg cholesterol. Although the Association of Official Analytical Chemists (AOAC) has recommended a method for cholesterol determination in egg yolk (AOAC, 1990), several methods are used in the literature. Most of the procedures used to determine the cholesterol content of foods are those originally developed for serum cholesterol with minor modifications. Serum cholesterol assay methods can be divided into three groups: colorimetric, chromatographic, and enzymatic procedures. All of these methods are presently available for the determination of cholesterol in eggs and are presently being evaluated for precision, accuracy, and recovery. The classical colorimetric methods for cholesterol determination are based on the Liebermann–Burchard color reaction or the Zlatkis procedure (Zak, 1977).

These methods have commonly been used in the past to determine the cholesterol content of eggs, however, the accuracy and the limitations of these procedures has been questioned for some time (Brown, 1961; Sweeney and Weihrauch, 1976; Naber and Biggert, 1985; Beyer and Jensen, 1989). Colorimetric methods, although accurate in determining cholesterol in purified samples, appear to overestimate significantly the amount of cholesterol in egg yolk when compared with gas chromatography (Marshall *et al.*, 1989; Bohac, 1988). To overcome these disadvantages, chromatographic techniques are now being used widely for cholesterol analysis in eggs. Chromatographic techniques involve the use of gas–liquid chromatography (Fenton and Sim, 1991), high performance liquid chromatography (Ulberth and Reich, 1992), and capillary supercritical fluid chromatography (Novak *et al.*, 1991). Usually a chromatographic cholesterol determination would include either direct saponification or indirect saponification of the

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samples prior to analysis. Indirect saponification would include the following lengthy steps:

1. extraction of the total lipids with an organic solvent or solvent mixture;
2. removal of the solvent;
3. alkali saponification of the total lipids;
4. extraction of the nonsaponifiable components with an organic solvent;
5. removal of the solvent;
6. derivatization of the unsaponifiable matter; and
7. chromatographic estimation of the analyte.

In direct saponification, egg yolk is saponified in alkali without the prior extraction of the total lipids. All these sample pretreatment steps are time-consuming, cumbersome, not environmentally friendly and limit the sample throughput. Enzyme preparations containing peroxidase, cholesterol esterase, and cholesterol oxidase have been employed satisfactorily to determine cholesterol in egg yolk (Shen *et al*., 1982; Jiang *et al*., 1990; Van Elswyk *et al*., 1991). However all these procedures, like gas chromatography, included either direct or indirect saponification of the yolk samples prior to cholesterol determinations, making this method less attractive. Nonetheless, cholesterol in egg yolk can be determined enzymatically and, by appropriate selection of conditions, the procedure can be made quick, efficient and environmentally safe. The Sigma Diagnostic Cholesterol Reagent measures cholesterol enzymatically and is a modification of the method of Allain *et al*. (1974). This reagent was developed for use on blood serum. Enzymatic reactions involved in this procedure are as follows:

$$\text{Cholesterol esters} + H_2O \xrightarrow[\text{esterase}]{\text{cholesterol}} \text{cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow[\text{oxidase}]{\text{cholesterol}} \text{cholest-4-en-3-one} + H_2O_2$$

$$2H_2O_2 - \text{Aminoantipyrine} + p\text{-hydroxybenzenesulfonate} \xrightarrow[]{\text{peroxidase}} \text{quinoneimine dye} + 4H_2O$$

Cholesterol esters are first hydrolyzed by cholesterol esterase (EC 3. 1 1.13) to cholesterol. The cholesterol produced by hydrolysis is oxidized by cholesterol oxidase (EC 1.1.3.6) to cholest-4-en-3-one and hydrogen peroxide (H_2O_2). The hydrogen peroxide produced is then coupled with a chromogen, 4-aminoantipryine and *p*-hydroxybenzenesulfonate, which in the presence of peroxide (EC 1.11.1. 7) yields a quinoneimine dye which has an absorbance maximum of 500 nm. The intensity of the color produced is directly proportional to the total cholesterol in the sample. Cholesterol reagent kits are convenient and relatively inexpensive and available for the quantitative enzymatic determination of total cholesterol concentration in serum or plasma.

In the following experiments we describe a method for determination of total cholesterol in fresh, frozen, and dried egg yolk using Sigma diagnostic cholesterol reagent without the need for saponification. This method involves solubilization with aqueous NaCl, a one-step sample preparation for simple, accurate and rapid quantification of cholesterol. The effectiveness of solubilization of yolk vs saponification of yolk to determine egg yolk cholesterol values was examined. The performance of this method was also tested by using an egg yolk reference material and by comparison with GC.

MATERIALS AND METHODS

Sample preparation

Eggs were obtained from local supermarkets. Sixty large eggs were divided into three groups of 20 each. Egg yolks from two groups were completely separated from the albumen, adhering white and chalazae; they were weighed, pooled and mixed thoroughly with a glass rod within each group. One group (fresh) was analyzed for cholesterol content immediately. The other group (frozen) was kept frozen at $-20^{\circ}C$ until analyzed. The third group of eggs (dried) were hard boiled for 5 min, their yolks were separated, pooled and mixed thoroughly, then air-dried overnight in a fume cupboard. The dried-matter content of the dried sample was determined by taking subsamples in triplicate and drying at $110^{\circ}C$ overnight in a forced air oven. The National Institute of Standards and Technology, Washington, DC provided a standard reference material (No. 1845) of cholesterol in whole egg powder. The cholesterol concentration of this standard, as determined by a modification of the isotope dilution-mass spectrometric definitive method for cholesterol, was 19.0 ± 0.2 mg cholesterol per g yolk. This standard was used as a control for the determination of inter-assay variation in cholesterol extraction values.

Saponification vs solubilization

Egg yolks from each group were saponified directly with 10 ml of alcoholic KOH solution (9.4 ml 95% ethanol plus 0.6 ml 33% KOH) in tightly capped tubes in a $60^{\circ}C$ water bath for 1 h, with occasional shaking. After cooling to room temperature, unsaponifiable fractions containing cholesterol were extracted with hexane and dissolved in dimethylformamide (DMF) and derivatized for 30 min using hexamethyldisilazane and trimethylchlorsilane to form the trimethylsilylester (AOAC Method, No. 43.290, 1984). 5-α-cholestane (Sigma Chemical Co., St Louis, MO, USA) was used as an internal standard (IS). Solubilization of the egg yolks from each group was performed by dispersing 1.5 g of

dry sample or 3 g of liquid yolk in 27 ml of 0.85% (0.15 M), 2% (0.32 M) and 5% (0.86 M) w/v NaCl solution, respectively. Samples were then gently shaken at room temperature for approximately 2 h in a tightly capped flask to prevent evaporation. A 1 ml solubilized yolk sample was further diluted 10-fold with the same concentration of NaCl solution and used as a working sample. Water was used in yolk solubilization as a control (no treatment).

Enzyme method

A commercial test kit for cholesterol analysis (Sigma diagnostic cholesterol reagent procedure No. 352, Sigma Chemical Co., St Louis, MO, USA) was used. The sample contained 0.1 ml of the chosen concentration of NaCl (i.e. 85, 2 or 5% (w/v)), 1 ml enzyme reagent and 0.01 ml of the cholesterol sample, either standard or unknown. A blank was prepared by substituting 0.01 ml of deionized water for cholesterol sample. The final volume of each reaction mixture was 1.11 ml. Samples were vortexed, then incubated for 15 min in a water bath at 37°C. Absorbances were read at 500 nm using a spectrophotometer (Shimadzu UV-160; Shimadzu Scientific Instruments, Columbia, MD). All samples were run in triplicate.

Gas chromatography

A Varian model 3700 equipped with flame ionization detector and on-column injector and a 30 m×0.53 mm ID capillary column packed with DB-1 (JandW Scientific, Folsom, CA) was used. Gas chromatography parameters were adjusted to retention times of 5 min for the internal standard peak (5-α-cholestane) and 10 min for cholesterol peak. The column temperature was 270°C isothermal, detector temperature was 300°C, and the injection port temperature was 280°C. Helium was the carrier gas at a flow rate of 10 ml min^{-1} with make-up gas at a flow rate of 20 ml min^{-1}. Cholesterol was quantified by reference to the integral of the internal standard peak. A Varian SP 4290 integrator (Varian Associates, Inc., Sunnyvale, CA) was used to integrate peak areas.

Calculation and statistical analysis

The amount of cholesterol was determined from gas chromatographic data by comparison of the integral of the cholesterol peak with that of the internal standard (5-α-cholestane) using appropriate response factors. The cholesterol concentrations of yolk samples were expressed as mg cholesterol per gram of fresh egg yolk.

On each of four separate days, egg samples were taken and saponified and then the batch for that day was analyzed three times by gas chromatography and by the enzyme method. For the other two methods, solubilization and no treatment (control), no saponification was required, so three samples of egg were taken from stock, solubilized and analyzed for cholesterol by the enzyme method on that day. In this way, there were three values per day for each method and experiments were performed on four separate days. For initial analysis, mean values of the three analyses for each method were taken for each day. This yielded four means per method, one for each day. These were analyzed by ANOVA to determine whether there were significant differences between the mean values for the various methods (means of the four daily means). For a second method of analysis, rather than take means of the three values per day, all 12 values per method (3 per day×4 days) were used for the ANOVA, giving a more powerful test. With the saponification, the first analysis is more appropriate, as each day with its separate saponification can be viewed as an experimental unit. Yet for the solubilization this is not so, because each of the 12 analyses was merely a sampling from the same batch. Because the aim here was to demonstrate equivalence of cholesterol values between saponification and solubilization using the enzyme method, Type I error was not a major concern; Type II error was more important. Thus, the results of the more powerful second analysis are quoted in this discussion. However, the first analysis generally yielded the same conclusions although trends were not always as significant, as would be expected from a less powerful analysis.

RESULTS AND DISCUSSION

The effect of solubilization with different NaCl concentrations on the cholesterol value of the egg powder standard, fresh, frozen and dried egg yolk as determined by the enzyme method are presented in Table 1. Results showed that for all egg preparations, at 2 and 5% (w/v) salt concentrations, cholesterol values were significantly higher than that at 0 and 0.85% (w/v) NaCl. There were no significant differences observed between 0 and 0.85% (w/v) or between 2 and 5% (w/v) salt concentrations. These results suggest that egg yolk components were not solubilized at low NaCl because of the initial ionic strength of egg yolk. Grodzinski (1951) reported that initial yolk is isotonic with 0.16 M NaCl solutions.

Table 1. Effect of solubilization with different NaCl concentrations on cholesterol value of egg powder standard, fresh, frozen, and dried egg yolk, as determined by enzyme method

Preparation	Cholesterol (mg per gram wet yolk)			
	Salt concentration (% w/v)			
	0	0.85	2	5
Egg powder standard	18.1[a]	18.2[a]	19.1[b]	19.0[b]
Fresh	11.8[c]	11.9[c]	12.5[d]	12.4[d]
Frozen	11.5[e]	11.4[e]	12.0[f]	12.0[f]
Dried	11.0[g]	11.1[g]	11.6[h]	11.6[h]

Values with different superscripts are significantly different ($P < 0.001$).

Table 2. Cholesterol content of egg powder standard, fresh, frozen and dried egg yolk as determined by GC analysis and the enzyme method with direct saponification and solubilization

Preparation	Cholesterol (mg per gram wet yolk)			
	GC, saponified	Enzyme, saponified	Enzyme, solubilized	Enzyme, no treatment
Egg powder standard	19.1[a]	19.4[a]	19.2[a]	18.0[b]
Fresh	12.4[c]	12.4[c]	12.6[c]	11.7[d]
Frozen	12.1[e]	11.9[e]	11.9[e]	11.4[f]
Dried	11.7[h]	11.3[g]	11.5[gh]	11.0[c]

Values with different superscripts are significantly different ($P < 0.05$).

However, the response of yolk solubility at the 2% level was probably caused by rupturing of yolk granules by salt (Burley and Cook, 1961), in turn making more cholesterol available for easy detection.

The cholesterol content of egg powder standard, fresh, frozen, and dried egg yolk as determined by gas chromatography and the enzyme method with saponification and solubilization is presented in Table 2. In this experiment, solubilization of all samples was carried out at 2% NaCl (w/v). The primary result is that for all egg preparations subjected to saponification and solubilization, cholesterol values were not significantly different using the enzyme method. Even at $P = 0.2$, significant differences were not found for dried and frozen product; for the standard and fresh product they were found but were inconsistent. When the enzyme method was used with saponification or solubilization, the cholesterol values generally agreed with the gas chromatography method. The exception was a higher cholesterol value, obtained from the gas chromatography method for the dried egg yolk. This value was only just significantly higher and did not represent a consistent trend over the different egg preparations; it is hypothesized to be a chance occurrence. For all egg preparations, cholesterol values obtained from untreated yolks were significantly lower ($P < 0.001$) than the other values. This procedure is not recommended because it significantly underestimates the cholesterol content.

A two factor ANOVA indicated that the means for fresh, frozen and dried eggs taken over all methods (12.3, 11.8, 11.4, respectively) were all significantly different ($P < 0.001$). It is possible that the 20 eggs chosen randomly from the same source for each processing method just happened to have different cholesterol levels. However, it is preferable to hypothesize that freezing lowers the detectable cholesterol level below that of fresh eggs, while drying lowers it further. This result is consistent with reports that the changes to egg yolk upon spray-drying are similar to those caused by freezing; namely aggregation and insolubilization of the low-density lipoprotein (Seideman and Cotterill, 1969). This is to be expected because lipoproteins owe part of their stability to hydrophobic interactions which require the presence of liquid water. Since 83% of yolk cholesterol is weakly bound to the low-density lipoproteins, as with freezing, the drying of yolk is expected to affect the level of detectable cholesterol.

The enzymatic method has been proven up to now to be useful in the determination of egg yolk cholesterol when saponified (Shen *et al.*, 1982). However, data obtained in the present study demonstrate that the enzymatic method can be used for the quantitative determination of cholesterol without the need for saponification. Solubilization of the samples can substitute for saponification. Solubilization of samples provides excellent accuracy and precision for the determination of yolk cholesterol using the enzyme method. This technique, with its excellent reproducibility, accuracy, and speed should be the choice for egg cholesterol determination, especially when a large number of samples are analyzed. Whether this method can be applied to the determination of cholesterol in other foods requires further investigation.

REFERENCES

Allain, C. C., Poon, L. S., Chan, C. S. G., Richmond, W. and Fu, P. C. (1974). Enzymatic determination of total serum cholesterol. *Clinical Chemistry*, **20**, 470–475.

AOAC (1990). Method 43.290. *Official Methods of Analysis of the AOAC*, 15th edn. Association of Official Analytical Chemists, Washington, DC.

Beyer, R. H. and Jensen, L. S. (1989). Overestimation of the cholesterol content of eggs. *Journal of Agricultural and Food Chemistry*, **37**, 917–920.

Bohac, C. E. (1988). Assessment of methodologies for colorimetric cholesterol assay of meats. *Journal of Food Science*, **53**, 1642–1644.

Brown, W. (1961). Errors in the determination of serum cholesterol. *Australian Journal of Experimental Biology and Medical Science*, **398**, 209–222.

Burley, R. W. and Cook, W. H. (1961). Isolation and composition of avian egg yolk granules and their constituent δ- and β-lipoproteins. *Canadian Journal of Biochemistry and Physiology*, **39**, 1295–1307.

Fenton, M. and Sim, J. S. (1991). Determination of egg yolk cholesterol content by on-column capillary gas chromatography. *Journal of Chromatography*, **540**, 323–329.

Grodzinski, Z. (1951). The yolk spheres of the hen's egg as osmometers. *Biological Reviews of the Cambridge Philosophical Society*, **26**, 253–264.

Jiang, Z., Cherian, G., Robinson, F. E. and Sim, J. S. (1990). Effects of feeding cholesterol to laying hens and chicks on cholesterol metabolism in pre- and posthatch chicks. *Poultry Science*, **69**, 1694–1701.

Marshall, M. W., Clevidence, B. A., Thompson Jr., R. H. and Judd, J. T. (1989). Problems in estimating amounts of food

cholesterol: three methods for mixed diets. *Journal of Food Composition and Analysis*, **2**, 2–12.

Naber, E. and Biggert, M. (1985). Analysis for and generation of cholesterol oxidation products in egg yolk by heat treatment. *Poultry Science*, **64**, 341–347.

Novak, R. A., Reightler, W. J., Pasin, G., King, A. J. and Zeidler, G. (1991). Supercritical fluid extraction of cholesterol from liquid egg. In *Fats and Cholesterol Reduced Foods Technologies and Strategies*, Vol. 12, ed. C. Haberstroh and C. H. Morris. Gulf Publishing Company, Houston, pp. 289–298.

Seideman, W. E. and Cotterill, O. J. (1969). Ion-exchange chromatography of egg yolk: heating, freezing, and spray drying. *Poultry Science*, **48**, 894–900.

Shen, C. J., Chen, I. S. and Sheppard, A. L. (1982). Enzymatic determination of cholesterol in egg yolk. *Journal of Association of Official Analytical Chemists*, **65**, 1222–1224.

Sweeney, J. and Weihrauch, J. (1976). Summary of available data for cholesterol in foods for its determination. *CRC. Critical Review Food Science and Nutrition*, **8**, 131–159.

Ulberth, F. and Reich, H. (1992). Gas chromatography determination of cholesterol in processed foods. *Food Chemistry*, **43**, 387–391.

Van Elswyk, M. E., Schake, L. S. and Hargis, P. S. (1991). Research Note: Evaluation of two extraction methods for the determination of egg yolk cholesterol. *Poultry Science*, **70**, 1258–1260.

Zak, B. (1977). Cholesterol methodologies: A review. *Clinica Chemica Acta*, **23**, 1201.