GLC Determination of Cholesterol as an Index of Egg Content in Baked Goods[1]

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ABSTRACT

A specific, quantitative determination of cholesterol in baked goods has been developed for use as an index of egg content. The unsaponifiable residue, which contains cholesterol and other sterols, if present, is quantitatively extracted from the baked product. All sterols present are derivatized to form their trimethylsilyl (TMS) ethers. Cholesterol, as its TMS derivative, is quantitatively determined by gas-liquid chromatography using 5-α-cholestane as an internal standard. Egg content of the baked product is calculated by comparison of the amount of cholesterol determined by the method, subject to any correction necessary for the presence of non-egg cholesterol, with an average value for the cholesterol content of eggs.

Egg contents of prepared foods have been determined by several methods using quantitative determinations of total lipids P_2O_5 (1), certain characteristic proteins of eggs (2), and total sterols (3) as indices. Of these methods, total lipid P_2O_5 is perhaps most limited in application due to interference of other phosphorus-containing lipid ingredients present in many foods. The quantitative determination of certain egg proteins by electrophoresis was used by Silano et al. (2) in 1968 as an index of the egg content of noodles. The latter method, however, may be limited in wider application because of possible protein interaction of denaturization during processing.

Egg contents of noodles have also been determined using total sterols as an index (3). The Bromination or Digitonin methods, AOAC Sections 14.140 and 14.141, respectively, 11th Ed. (4), both of which are lengthy and involved, have been official methods of the AOAC for determining total sterols in noodles for a number of years. More recently, Roberts (5) reported a simpler, more rapid fluorometric method for determining total sterols in noodles for the purpose of calculating egg content. The fluorometric method, Section 14.144, is now also an official method of the AOAC (4).

Inasmuch as cholesterol is the only quantitatively significant sterol present in eggs, the use of total sterols as an index of egg content of foods is limited to those cases such as egg noodles where corrections for sterols other than cholesterol can be made, and where non-cholesterol sterols are absent or present only in insignificant quantities. It would appear, therefore, that a specific determination for cholesterol would have definite advantages for use as an index of egg content.

Although cholesterol in food materials has been quantitatively determined by gas-liquid chromatography (GLC) as the free sterol (6) and as its acetate derivative (7), decomposition and/or column adsorption of the free sterol can cause inaccurate results (8), and the formation of the cholesterol-acetate derivative is rather complex and time consuming. The quantitative analysis of cholesterol as its trimethylsilyl (TMS) derivative by GLC, using 5-α-cholestane as an internal standard,

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which has been quite widely reported in biochemistry and chromatography journals (e.g., 8-11), has been shown to be considerably more accurate than chromatographing the free sterol, by virtual elimination of the difficulties encountered with the latter (8). Moreover, we have found that the formation of the cholesterol-TMS derivative can be achieved much more rapidly and simply than the formation of cholesterol acetate.

This paper reports the development of a specific, quantitative GLC determination of cholesterol in baked goods as its TMS derivative, for use as an index of egg content. In addition to use as an index of egg content in a considerably wide range of other food products, the method should also find application in providing accurate dietary information on the cholesterol content of foods in general.

MATERIALS AND METHODS

Principle of Method

Cholesterol and other sterols, if present, are quantitatively extracted from baked or other food products as part of the unsaponifiable residues. All sterols present are derivatized to form their TMS ethers. Cholesterol, as its TMS derivative, is quantitatively determined by GLC using 5-*α*-cholestane as an internal standard. Egg contents of baked products are calculated by comparison of the amounts of cholesterol found by the above method with an average value for the cholesterol content of eggs.

Following is a detailed outline of the procedures and calculations used for the cholesterol and egg-content determinations reported in this paper.

Isolation of Unsaponifiable Residue

To isolate unsaponifiable residues, the following methods were used: for eggs, AOAC procedure 17,018 (4); for baked products and flour, NFDM-AOAC procedure 14,139 (4); and for shortenings, AOAC procedure 28.063, using a 5-g. sample (4).

All AOAC procedures were followed, up to and including the removal of H_2O from the separatory funnels after the final rinse. The ether solutions were then filtered through glass funnels containing small pledgets of glass wool and approximately 15 g. anhydrous Na_2SO_4 into 250-ml. Erlenmeyer flasks to remove the remaining H_2O. The separators and funnels were rinsed with several small (approximately 10-ml.) portions of ethyl ether into the 250-ml. Erlenmeyers.

The ether was evaporated to dryness on a steam bath and the unsaponifiable residues dissolved in warm (approximately 70°C.) N,N-dimethylformamide (DMF) and transferred quantitatively to volumetric flasks. (Final room temperature volumes were 25 ml. for eggs and butter, and 10 ml. for baked goods and all other samples.)

Cholesterol and 5-*α*-Cholestane Standards

The following were used as standards: 1) cholesterol stock solution: 100 ml. DMF solution containing 10 mg. cholesterol per ml.; 2) cholesterol working standard: 100 ml. DMF solution containing 0.5 mg. per ml.; and 3) 5-*α*-cholestane internal standard: 100 ml. *n*-heptane solution containing 0.5 mg. per ml.

Both cholesterol and 5-*α*-cholestane were obtained from Mann Research Laboratories, New York, N.Y.

Preparation of Derivatives

Trimethylsilyl derivatives were prepared by a modification of the procedure reported by Sweeley et al. (12) for carbohydrates. Two-tenths milliliters hexamethyldisilazane (HMDS) (Matheson Coleman & Bell, East Rutherford, N.J.) and 0.1 ml. trimethylchlorsilane (TMCS) (General Electric Co., Silicone Products Div., Waterford, N.Y.) were added to separate 1-ml. aliquots of unsaponifiable residue solutions and cholesterol working standard solution in 15-ml. conical glass-stoppered centrifuge tubes, the tubes then being shaken vigorously for 15 to 20 sec. After the reaction mixtures were allowed to stand undisturbed for 15 min., 1 ml. of 5-*α*-cholestane internal standard solution followed by 10 ml. of water was added to each tube. The tubes were again shaken vigorously for 1 min. and then centrifuged for 1 min. at approximately 2,000 r.p.m. The end result was a two-phase system consisting of a lower water-DMF layer, and an upper *n*-heptane layer containing sterol-TMS derivatives and the 5-*α*-cholestane internal standard.

The presence of excessive moisture interferred with the silylation reaction and caused incomplete derivatization of cholesterol and other sterols to their TMS ethers. For this reason it was necessary that the DMF solutions of samples and cholesterol standards were essentially free of H_2O prior to derivatization. DMF specified to contain a maximum of 0.1% H_2O was found to give satisfactory results. Once the silylation reaction was complete, however (found to be within 15 min.), all sterol-TMS derivatives were stable in the presence of water. The addition of water at this point in the method enabled the sterol-TMS derivatives to be completely extracted into the *n*-heptane layer containing the 5-*α*-cholestane internal standard, both the latter and the sterol-TMS derivatives being insoluble in the DMF-water layer. Although GLC analyses generally were run on the same day the samples were derivatized, the derivatives appeared to be stable in *n*-heptane for at least a week and probably longer. Addition of the *n*-heptane solution alone to the DMF also produced a two-phase system, but GLC results indicated that the extraction of the sterol-TMS derivatives into the *n*-heptane layer was not complete when the addition of water was omitted.

Attempts to use an all-DMF solvent system in which the sterol-TMS derivatives and 5-*α*-cholestane internal standard were injected into the chromatograph in DMF solution had two disadvantages. One was that 5-*α*-cholestane is only very slightly and with difficulty soluble in DMF. As a result, even at maximum solubility, the concentration of 5-*α*-cholestane in DMF was considered too small for practical use as an internal standard. The second disadvantage was that DMF tailed excessively when chromatographed and interfered with the 5-*α*-cholestane peak. Extraction of the sterol-TMS derivatives into *n*-heptane after addition of H_2O eliminated the tailing problem encountered with an all-DMF solvent system.

Gas-Chromatographic Analysis

One-microliter samples were taken from the *n*-heptane layers of derivatized samples and standard cholesterol, respectively, and chromatographed under the following conditions: instrument, Varian Aerograph Model 1520B; column, 5′ × 1/8″ O.D. stainless steel, 5% QF-1 on 80/100 mesh Gas Chrom Q; detector, flame ionization; injector and detector temp., 270°C.; column temp., 230°C., isothermal; carrier gas flow, 32 cc. per min. (helium); sensitivity, range 10, attenuation 2.

During most of the determinations an Infotronics Model CRS-100 digital integrator was used to measure peak areas. Otherwise, peak height was used as a measure of chromatographic response.

Calculations

Cholesterol content was calculated as follows:

$$\text{\% cholesterol, total solids basis} = \frac{(\text{R chol. sam./R int. std.}) \times \text{wt. chol. std.} \times 10{,}000}{(\text{R chol. std./R int. std.}) \times \text{wt. orig. sam.} \times (100-M)}$$

Where R = chromatographic response consistently expressed in either peak area or peak height units; chol. sam. = sample cholesterol-TMS derivative; chol. std. = standard cholesterol-TMS derivative; int. std. = 5-α-cholestane internal standard; wt. chol. std. = weight in mg. of cholesterol standard taken for derivatization; wt. orig. sam. = weight in mg. in terms of original sample taken for derivatization; and M = % moisture in food product.

Egg content of baked goods was calculated as follows:

$$\text{\% egg, as whole dried egg solids, total solids food products basis} = \frac{\text{\% Cholesterol, total solids basis} \times 100}{A}$$

Where A = average % cholesterol content of whole dried egg solids, or actual % cholesterol content of eggs, on a total solids basis, used to make the baked product.

RESULTS AND DISCUSSION

Gas-Chromatographic Response

Under the GLC conditions employed, retention times of 2.6 and 5.0 min. were obtained for 5-α-cholestane and the TMS-cholesterol derivatives, respectively. Other sterol-TMS derivatives, if present, were well separated from the other components. Figure 1 shows three typical chromatograms of *n*-heptane solutions containing derivatized cholesterol obtained from different sources – a cholesterol standard, an egg extract, and an extract of a cake baked with egg. All three solutions contained the 5-α-cholestane internal standard. Some unknown components were carried through the extraction and derivatization of the egg and cake samples, but were eluted with the solvent front and did not interfere with the peaks of interest. The phytosterols campesterol and β-sitosterol in chromatogram C originated in the flour used in the cake formula.

A glass tube liner[2] was used in the injection port of the chromatograph and served to prevent decomposition of sterol-TMS derivatives which otherwise sometimes occurred if the derivatives were permitted to come into contact with the hot metal surfaces of the flash heater. Apparently the critical area with respect to sample decomposition was the injection port and not the column itself, inasmuch as stainless-steel columns were used successfully.

[2]As suggested in "Handbook of Silylation GPA-3", p. 7, Pierce Chemical Co., Rockford, Ill., 1970.

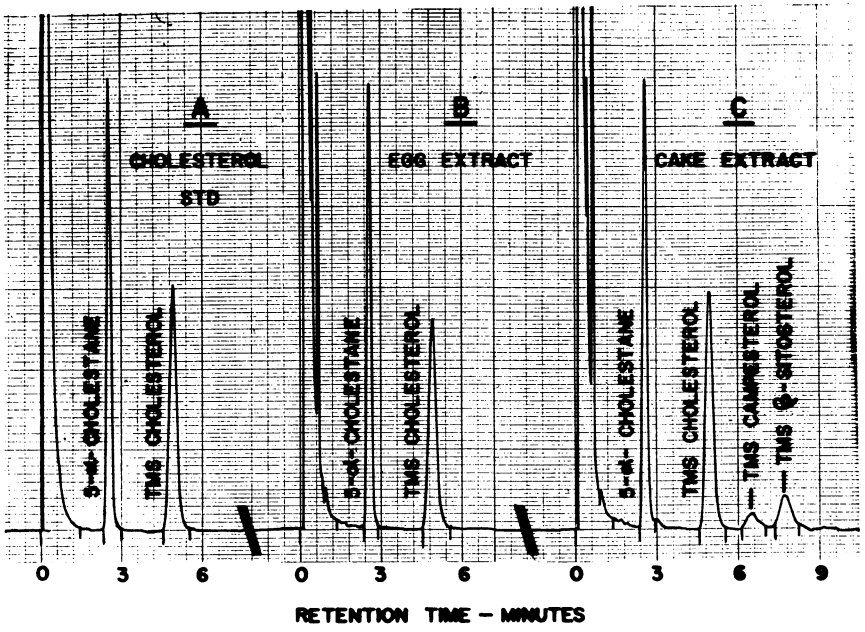

Fig. 1. Chromatograms of *n*-heptane solutions containing cholesterol obtained from three different sources.

Chromatographic response was found to be linear over a fairly wide range of TMS-cholesterol concentration. A regression analysis of a plot of concentration of cholesterol in terms of mg. per ml. of DMF solution vs. peak area ratio TMS-cholesterol/5-*α*-cholestane over a concentration range of 0 to 2.0 mg. cholesterol per ml. indicated the correlation coefficient to be 0.99. Additional visually plotted points on the curve obtained with higher concentrations of cholesterol (but not subjected to regression analysis) indicated that response was linear up to at least 4 mg. per ml. In the analyses of eggs and baked products for cholesterol content, the linear range of 0 to 4 mg. cholesterol per ml. proved to be more than adequate. With the sample sizes and final volumes of DMF solution indicated in the method, about 0.6 mg. cholesterol per ml. DMF solution was obtained in the case of whole dried eggs, and 0 to about 1 mg. per ml. in the case of the baked products analyzed.

GLC peak-area ratios of derivatized 0.5 mg. per ml. cholesterol standard to the 0.5 mg. per ml. 5-*α*-cholestane internal standard, calculated to the nearest 10^{-3}, were consistently near unity. The standard deviation of average ratios obtained for seven separately derivatized standards prepared on different days was 0.00518. The standard deviation of average ratios obtained for a series of five separately derivatized standards prepared on the same day was 0.00487. Each average ratio was calculated from at least four replicate GLC runs per separately derivatized standard. The overall average standard deviation for replicate ratios obtained within a GLC run was 0.00332.

These data indicate that a given amount of cholesterol can be determined

quantitatively by GLC as its trimethylsilyl-ether derivative with good precision, and that the derivative can be prepared with slightly lower but entirely acceptable precision. The fact that slightly better precision was obtained for cholesterol standards derivatized on the same day as opposed to those derivatized on different days was attributed to daily cleaning of the flame ionization detector necessitated by the build-up of silica deposits which tended to cause "noise" and decreased overall sensitivity. Evidently, slight repositioning of the ignitor electrode with respect to the flame tip, which occurred during cleaning, caused slight variations in relative response of the TMS-cholesterol derivative and internal standard. In order to compensate for the latter and other slight variations in relative response brought about by any day-to-day changes in chromatographic conditions, as well as any possible change in concentration of the 5-*α*-cholestane internal standard due to evaporation of *n*-heptane prior to derivatization during storage, cholesterol standards were derivatized and chromatographed each day egg or baked-product sample extracts were derivatized and analyzed for cholesterol content.

Average Cholesterol Content of Eggs

Aside from the contribution of non-egg cholesterol in certain foods, the accuracy with which egg content of foods can be determined using cholesterol as an index is largely dependent on the range of cholesterol content found in eggs. As indicated by Stauffer (13) in 1967, the average cholesterol content of eggs was found to have decreased during the past 20 years. Table I lists the results of

TABLE I. CHOLESTEROL CONTENT OF 14 WHOLE DRIED EGG SAMPLES, TOTAL SOLIDS BASIS

Source[b]	% Cholesterol	Source[b]	% Cholesterol
N.Y.	1.64	N.Y.	1.68
Oreg.	1.66	N.Y.	1.76
Oreg.	1.67	Oreg.	1.77
Tenn.	1.67	N.Y.	1.77
Wash., D.C.	1.67	N.Y.	1.78
Mass.	1.68	Wash., D.C.	1.78
Mass.	1.68	Tenn.	1.81

Average percentage of cholesterol: 17.2; standard deviation: 0.058.

[a]Bakery location.

cholesterol determinations run on 14 whole dried egg samples obtained from various sections of the U.S. for the purpose of providing up-to-date data for calculating an average value for the cholesterol content of eggs. As indicated, the average value was 1.72%. Based on the standard deviation of 0.058, at a 95% confidence level, the expected range of the average value would be 1.68 to 1.76%, and the expected range for any one given egg sample would be 1.57 to 1.87%. It should be emphasized that the latter data and the results given in Table I do not necessarily reflect those obtainable on a truly nationwide basis, which would require the analysis of a larger number of samples from a larger number of different sections of the U.S.

The data obtained on the 14 egg samples, however, suggest that the use of 1.72% as an average value for cholesterol in eggs in calculating egg content of food products would enable results to be obtained within approximately ±10% of the true content, assuming 100% recovery of all cholesterol present, and that the eggs were the only source of cholesterol in the food product. In most cases, however, the use of the 1.72% average should enable more accurate results to be obtained. The ±10% figure is based on the projected low and high cholesterol content of eggs of 1.57 to 1.87%. It is expected that the actual cholesterol content of most eggs would be closer to the 1.72% average determined.

Precision and Accuracy of Overall Cholesterol Determination

Table II lists the results of a replicate analysis of one whole dried egg sample for cholesterol content in which five separately weighed replicates were saponified and derivatized. The low standard deviation and low coefficient of variation indicate that the overall cholesterol determination, which includes saponification and extraction as well as GLC analysis, also can be conducted with good precision.

As an indication of the degree of accuracy of the overall cholesterol determination, the percent recovery of known amounts of cholesterol added to the same whole dried egg sample which was subjected to replicate analysis was determined. Cholesterol in ethanol solution was added prior to saponification at two levels in duplicate, equivalent to 12.5 and 25.0 mg. per g. of total mixture (added cholesterol and egg), respectively. The average recovery at each level was 99.82 and 98.20%, respectively. The latter results were based on the average cholesterol content of the egg sample determined by the replicate analysis.

TABLE II. REPLICATE ANALYSIS OF ONE WHOLE DRIED EGG SAMPLE FOR CHOLESTEROL CONTENT, TOTAL SOLIDS BASIS

Sample No.	% Cholesterol[a]
1	1.675
2	1.682
3	1.669
4	1.691
5	1.676
Average	1.679
Standard deviation	0.00851
Coefficient of variation	0.5068

[a]Average of eight replicate GLC runs per sample.

TABLE III. DETERMINATION OF CAKE EGG CONTENT AS WHOLE DRIED EGG SOLIDS, TOTAL SOLIDS CAKE BASIS

	Determined	
Added	Actual egg cholesterol basis	Average egg cholesterol basis
0%	0%	0%
1.48	1.47	1.55
2.20	2.23	2.35
2.92	2.82	2.98
4.43	4.26	4.48

Accuracy of Method as an Index of Egg Content

Table III lists the results of the analysis for determination of egg content of a series of small "sponge" cakes containing known amounts of egg. No shortening was present in the formula for these cakes. Results were calculated both on the basis of the actual cholesterol content of the eggs used in the cakes, which in this case was found to be 1.81%, and on the 14-sample average basis, 1.72%. As expected, the results obtained on the average egg cholesterol basis were somewhat

higher than those obtained on the actual egg cholesterol basis. The fact that the 2.98 and 4.48% results obtained on the average egg cholesterol basis agree more closely with the amounts of egg added than the corresponding results obtained on the actual egg cholesterol basis presumably was due to loss of unsaponifiable matter during extraction, or possibly to a slight variation in scaling of ingredients. At any rate, however, all the results shown in Table IV would be acceptable from a practical standpoint.

TABLE IV. DETERMINATION OF HAMBURGER BUN EGG CONTENT AS WHOLE DRIED EGG SOLIDS, TOTAL SOLIDS BUN BASIS

	Determined	
Added	Actual egg cholesterol basis	Average egg cholesterol basis
0%	0%	0%
0.64	0.65	0.67
1.29	1.29	1.32
2.31	2.10	2.16

Under the GLC conditions employed for quantitative analysis, a peak having the same retention time as the TMS-cholesterol derivative was obtained for the zero-egg-added cake sample. The amount of cholesterol represented by the peak, however, was too small to affect the results, and was disregarded in the calculations. It is interesting to note that subsequent rechromatographing of the derivatized zero-egg-added cake sample extract on the same column at a higher sensitivity, lower column oven temperature (225°C.) and lower carrier gas flow rate (25 cc. per min.), revealed that the "TMS-cholesterol" peak actually consisted of two components. Whereas a single peak was obtained under the GLC conditions used for quantitative analysis, two partially separated peaks were obtained at the lower

A comparison of retention times of the two peaks in the derivatized zero-egg-added cake extract obtained under the latter GLC conditions was made with those obtained for a derivatized cholesterol standard and derivatized extracts of nonfat dry milk and the flour used in the cake formula. It was established that one of the two components in the zero-egg-added extract was TMS-cholesterol. The source of the cholesterol was traced back to the nonfat dry milk. Although the other component was not identified, its source was traced back to the flour, the derivatized extract of which contained the unidentified component, but no TMS-cholesterol. Derivatized extracts of two other samples of flour from different sources also revealed the presence of the same unidentified component, but no TMS-cholesterol was detected. Furthermore, no TMS-cholesterol was detected when the three derivatized flour extracts were chromatographed on a column containing a different stationary phase-JXR. The fact that we were unable to detect cholesterol in flour seems to be in conflict with results reported by Berry et al. in 1968 (14).

Table IV lists the results of analysis for the egg content of hamburger buns made

with known amounts of egg and vegetable shortening at a 6% level on a total solids basis. In this case, due to the relatively small difference between the actual and average egg cholesterol content – 1.77 vs. 1.72% – there is closer agreement between the results obtained on the two bases, as opposed to the cake egg content analysis results (Table III). As with the cake analysis, the zero-egg-added hamburger bun extract exhibited a peak having the same retention time as TMS-cholesterol when chromatographed under the conditions used for quantitative analysis. The sources of the peak were found to be traces of cholesterol in the nonfat dry milk and the vegetable shortening used, plus the unknown, noncholesterol component in flour. Again, the size of the peak was too small to affect the results.

The results given in Tables III and IV indicate that the overall method is sufficiently accurate and valid for determining the egg content of baked goods, providing that egg is the only significant source of cholesterol. The use of other ingredients containing cholesterol, such as animal fats and certain dairy products would tend to cause high results to be obtained.

TABLE V. HYPOTHETICAL EFFECT OF NON-EGG CHOLESTEROL ON HAMBURGER BUN EGG CONTENT ANALYSIS

	"Determined"	
Egg Added	6% Lard	6% Butter
0%	0.33%	0.94%
0.64	0.97	1.57
1.29	1.62	2.22
2.31	2.63	3.22

Table V shows the hypothetical effect of the use of lard and butter, both at a 6% level on a total solids basis, instead of vegetable shortening, on the egg content analysis of hamburger buns. These results were calculated on the basis of 0.09% cholesterol in lard and 0.25% cholesterol in butter, both experimentally determined in typical samples by the present method. The 1.72% average cholesterol content of eggs was used in the calculations of "Determined" egg contents of the baked product, 100% recovery of all cholesterol being assumed. The effect of cholesterol from lard added at the 6% level is more pronounced at the relatively lower levels of egg content. Overall, however, and particularly at higher, functionally significant egg contents, the effect of the lard on egg content would be quite small and in most actual analyses could be disregarded.

The use of butter at a 6% level would produce larger errors, again the effect being more noticeable at lower egg contents. Depending on the degree of accuracy desired, in most actual analyses for egg content of baked products containing butter, a correction for the cholesterol contributed by butter would probably be necessary. Conceivably, the butter content in the baked product could be estimated from the fatty acid composition of the total lipid extract and a correction factor for non-egg cholesterol calculated accordingly. In many cases where a baked product is analyzed for egg content, however, label declarations would indicate the need for calculating any correction factor for non-egg cholesterol.

The determination of cholesterol by the present method could also be used as an index of egg content of such varied items as so called "egg extenders", egg noodles, pumpkin and custard pies, and donut and cake mixes. Egg content of foods may be expressed, where desired or necessary, as liquid whole eggs, or dried or liquid egg yolks, by multiplying the results determined as whole dried eggs by an appropriate factor. As previously mentioned, the method could also find application as a specific, accurate determination of cholesterol content of foods in general.

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