Selective Gas Chromatographic Determination of Cholesterol in Eggs

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A selective gas-chromatographic method is proposed for the determination of cholesterol in egg samples. The method consists of extraction of the lipid fraction and cold saponification prior to determination on a glass capillary column. The absence of cholesterol oxidation during the process was checked, and the conditions in which oxidation is avoided are discussed. The method also showed good precision (coefficient of variation = 1.39%). Applying this method to different fresh and frozen egg samples, we obtained a mean content of 392 mg of cholesterol/100 g of edible portion. These values are clearly lower than the majority of results published for eggs.

KEY WORDS: Cholesterol, cholesterol oxidation, eggs, gas-chromatographic determination.

The repercussions of diet and of its imbalance on human health is a priority in medical research, and it is also an area of growing interest to consumers. However, it is sometimes difficult to find objective and reliable data. The cholesterol content of eggs is the key factor in the assessment of their role in our diet. Many national and international organizations have recommended (1-3) decreasing total intake of cholesterol in developed countries, with the aim of reducing the incidence of coronary heart disease (CHD). In the last few decades, many studies on the influence of diet lipids on such diseases have been reported (4-11). Although a slight effect of cholesterol intake on plasma cholesterol levels and CHD incidence has been demonstrated, this effect is minimized by a well-balanced composition of lipids in the diet, which can be evaluated by the unsaturated/saturated ratio (12,13). Egg fat composition is well balanced (13,14), and some authors (15) report data that suggest that normal consumption of eggs does not increase plasma cholesterol levels.

However, the majority of these studies and recommendations have led to a certain "cholesterophobia" and, consequently, to a clear decrease in egg consumption in many developed countries. Thus, *per capita* consumption in Spain fell from 326 eggs/year in 1976 to 234 eggs in 1991. This decreasing consumption is also observed throughout the European Community (217 eggs/year in 1991) and in the United States (233 eggs/year in 1991) (16,17).

There is clearly a need for reliable cholesterol content data in foods. Cholesterol data available from published food composition tables show wide variability, mainly due to the differences in analytical methods used. Recent studies by Beyer and Jensen (18) showed that the colorimetric methods generally used in cholesterol determination in eggs give higher values (26–46%) than those obtained by gas chromatography (GC). Similar conclusions were reached by Ulberth and Reich (19) when they compared the cholesterol determination in foods by colorimetric and chromatographic methods.

So, it can be concluded that the cholesterol content in foods, and consequently cholesterol intake, is frequently overestimated and that its chromatographic determination should be considered more extensively. Thus, results obtained by Marshall *et al.* (20) in mixed diets show that the total cholesterol content in different kinds of diets, determined by GC, were about 75% of the values calculated for the same diets from food composition tables, and about 50% of colorimetric values.

The aim of this study was accurate determination of the cholesterol content in selected commercial samples of fresh and frozen eggs by a gas-chromatographic method. These data could be of interest in studies of cholesterol intake. The analytical procedure was characterized to ensure the best selectivity, accuracy and precision, and special attention was paid to oxidation of the cholesterol during the procedure.

MATERIALS AND METHODS

Samples. Cholesterol was determined in ten fresh egg and six frozen egg samples. The precision and recovery of the method was determined from one of the fresh samples. The contents of the fresh eggs were homogenized directly, and of the frozen samples after thawing, in a Ystral electric drive 10/20 3000 homogenizer (Scientific Instrument Centre Limited, Liverpool, United Kingdom), at 20,000 rpm for 1 min.

Reagents and standards. All solvents used were ACS-grade. Chloroform, methanol and diethyl ether were supplied by Panreac, Montplet & Esteban (Barcelona, Spain), and ethyl acetate was from Merck (Darmstadt, Germany).

Other reagents used were sodium hydroxide and anhydrous sodium sulfate (both for analysis) supplied by Panreac, dried pyridine (<0.01% water) from Merck and N,O-*bis*(trimethylsilyl)acetamide/trimethylchlorosilane/N-trimethylsilylimidazol, 3:2:3, for research (Sylon BTZ), in 0.1-mL glass ampoules, from Supelco, Inc. (Bellefonte, PA).

Standards of 5α-cholestane (98%) and cholesterol (>99%) were supplied by Sigma Chemical Co. (St. Louis, MO). These standards were weighed on a Sartorius 2004 MP microbalance (Göttingen, Germany) to an accuracy of 0.01 mg. These weighed standards were kept as ethyl acetate solutions in tightly sealed amber glass vials, at −20°C, for easier handling. The standard solutions were stored for a maximum of 1 mon after preparation.

Lipid extraction. The method proposed by Folch *et al.* (21) was followed, with some modifications, according to the procedure described here. About 0.6 g of fresh egg was weighed and homogenized in a conical flask (25 mL). Then, 2.5 mg of the internal standard (5α-cholestane) and 15 mL of a chloroform/methanol mixture (2:1, vol/vol) were added to the conical flask, and the mixture was stirred magnetically for 30 min. The liquid extract obtained was decanted on a paper filter and recovered in a tube (26 × 100 mm) with screw cap. The same chloroform/methanol mixture (10 mL) was added to the solid residue in the conical flask and agitated for 20 min. Then the blend was also passed through the same filter, and the conical flask and the paper were finally washed in 5 mL of the chloroform/methanol mixture. All the solvent fractions were recovered in the same tube.

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Distilled water (5 mL) was added to this tube, and after agitation, the blend was centrifuged for 20 min at 2,200 rpm. The chloroform phase was separated and removed with a Pasteur pipette and transferred to a round-bottom flask, from which the solvent was removed in a rotatory vacuum evaporator at 35°C. The evaporation was completed by a nitrogen stream and by keeping the flask in a vacuum desiccator for 30 min (approximately 10 mm Hg).

Cold saponification. Cold saponification was chosen to avoid cholesterol oxidation, in spite of the controversial nature of this decision. Bascoul *et al.* (22) affirm that 2 h of high-temperature saponification with 1N KOH does not involve cholesterol oxidation. In contrast, other authors (23,24) detect cholesterol oxides as a result of this kind of saponification, and they do not appear in cold conditions (25,26). Finally, Maerker and Unruh (23) also observed formation of cholesterol oxides in a method without saponification in the presence of phenolic antioxidants. In contrast, some recent studies support the accuracy of high-temperature saponification methods (27,28).

The procedure was as follows: Under low light conditions, 10 mL of 1N KOH solution in methanol was added to the flask containing the lipid extract, with gentle agitation to obtain a homogeneous phase. Then, the mixture was kept at room temperature for 20 h to complete the saponification. The blend was then quantitatively transferred to a tube (26 × 100 mm) with 10 mL of diethyl ether and 10 mL of distilled water. The sealed tube was then shaken, and after separation the organic phase was transferred to a separating funnel with a Pasteur pipette. The aqueous phase in the tube was extracted twice more with 2 portions of 10 mL of diethyl ether, and the new organic phases obtained were also transferred to the same funnel. This organic extract was washed in the funnel, first with 5 mL of 0.5 N KOH aqueous solution, and then with 2 portions of 5 mL distilled water. The washed organic extract was filtered through anhydrous sodium sulfate and recovered in a round-bottom flask, where the solvent was partially removed by means of a rotatory vacuum evaporator at 30°C. Removal of solvent was completed in a nitrogen stream and by keeping the flask in a vacuum desiccator for 30 min (approximately 10 mm Hg).

Gas chromatography. The nonsaponifiable extract in the flask was redissolved in 5 mL anhydrous pyridine, and 50 μL of this solution was transferred to a Pyrex microtube (10 × 75 mm). Sylon BTZ (50 μL) was added, and the mixture was kept at room temperature for 20 min to complete the silanization reaction. The derivatives were stable for several days at −20°C (29). All samples were injected in duplicate.

GC was performed on a Perkin-Elmer (Norwalk, CT) Autosystem, equipped with a flame-ionization detector and a fused-silica capillary column (25 m × 0.25 mm i.d.) with a film thickness of 0.13 μm stationary phase of methyl silicone (CP-Sil 5 CB; Chrompack, Middleburg, Holland). Helium was used as carrier gas.

Oven temperature programs were: (i) from 210 to 264°C at 2°C/min and 1 min at 264°C; (ii) from 210 to 290°C at 6°C/min and 2.7 min at 290°C. Injector temperature was 290°C, and detector temperature was 350°C. Split ratio was 1:30. Inlet pressure was 15 psi, and the sample volume injected was 2 μL.

Temperature program 1 was used in the determination of the selectivity of the method, and all other determinations were carried out by using program 2.

Cholesterol oxides' identification. Oxidation of cholesterol was determined by following a procedure similar to that proposed for the determination of cholesterol, with an additional step of purification by silica cartridge fractionation. This procedure, which we are developing for the determination of cholesterol oxides in eggs and egg products, will be published separately. By applying this method, we were able to establish that cholesterol oxides were not formed from the cholesterol standard (15 mg). For identification of cholesterol oxides, we used the following GC/mass spectrometry (MS) system: Hewlett-Packard 5890 gas chromatograph was used, equipped with a capillary fused-silica column (25 m × 0.32 mm i.d.), with a film thickness of 0.52 μm stationary phase of methyl silicone (Hewlett-Packard, Geneve, Switzerland). Oven temperature program was from 210 to 264°C at 2°C/min, from 264 to 290°C at 5°C/min, and 2 min at 290°C. Temperature of injection was 290°C and the carrier gas was helium, with an inlet pressure of 15 psi and a split ratio of 1:50. A Hewlett-Packard 5988A mass spectrometer was used with an interphase temperature of 280°C, an ion source temperature of 200°C and an electron energy of 70 eV. The cholesterol oxides were identified in the mass range (*m/z*) from 100 to 650, and the selected ion monitoring technique was also applied, in which the ions (*m/z*) 120, 129, 131, 321, 367, 382, 384, 403, 456, 472, 474 and 546 were selected as the most characteristic for the main oxides (7β-hydroxycholesterol, cholesterol α-epoxide, cholestantriol, 7-ketocholesterol and 25-hydroxycholesterol).

RESULTS AND DISCUSSION

Absence of cholesterol oxidation during the analytical procedure. Different ways have been proposed to avoid formation of cholesterol oxidation derivatives during the saponification step in cholesterol determination. Changes of the duration and temperature of the saponification and the addition of antioxidants are the most common modifications (18,19,23,25,30).

To check the absence of cholesterol oxidation as a result of our method, we first tested the purity of the cholesterol standard. Solutions of 0.8, 1.8 and 3.5 mg of cholesterol standard were prepared in 50 μL of pyridine, and 50 μL of Sylon BTZ was added to each one. After 20 min, they were injected onto the column, and no peak corresponding to cholesterol oxides was observed on the chromatograms. The cholesterol oxide limits of detection for the chromatographic determination, calculated according to the procedure of Knoll (31) and expressed as ng/g of the sample, were 7β-hydroxycholesterol, 40.3; cholesterol α-epoxide, 44.5; cholestantriol, 35.0; 7-ketocholesterol, 36.8; and 25-hydroxycholesterol, 29.0.

The method for identification of cholesterol oxides was then applied to some egg samples, in which we performed extraction of lipids and cold saponification as described above. Two conditions of evaporation of final extracts were tested: (i) total evaporation of the solvent to dryness at 45°C, and (ii) evaporation to a small volume of solvent at 30–35°C and complete evaporation to dryness by a nitrogen stream, followed by time in a vacuum desiccator. Under the first conditions, traces of some cholesterol

oxides were observed (7β-hydroxycholesterol and 7-ketocholesterol), while cholesterol α-epoxide, cholestantriol and 25-hydroxycholesterol were not formed. Under the second set of conditions, traces were not observed of any of the five cholesterol oxides studied.

So, we can conclude that oxidation of cholesterol can be avoided if some basic rules are observed, such as low-light conditions during the process; evaporation always performed under vacuum, at 30–35°C as the maximum temperature, and not to dryness.

Selectivity of the method. The selectivity was checked by using oven temperature program 1, which gave a higher resolution between the peaks. Increasing dilutions of the nonsaponifiable extract were prepared and injected onto the column to reveal the presence of possible peaks that appear near that of cholesterol, which could interfere with its determination. Figure 1A shows a chromatogram corresponding to the injection of the whole nonsaponifiable extract after silanization [25 μg internal standard (IS)]. Figure 1B shows the chromatogram corresponding to the injection of diluted extract, according to the procedure finally used for all samples (2.5 mg IS). The selectivity for cholesterol quantitation was highly improved with that dilution, because the second chromatogram only shows two peaks, corresponding to the IS and the cholesterol. In this way, the interfering peaks of the first chromatogram disappear and also do not affect the cholesterol peak area because of their different retention time.

Linearity of response and cholesterol relative response factor (RRF). The linearity of the response for cholesterol was determined by a calibration curve. Four repetitions of seven concentration levels were performed: 5, 10, 20, 30, 40, 50 and 60 μg of cholesterol, with 25 μg of 5α-cholestane added as IS. All solutions were prepared in 50 μL pyridine, and 50 μL of Sylon BTZ was added to each solution. After 20 min of reaction, they were injected into the chromatograph in duplicate.

The calibration curve of cholesterol is given by the regression of variable y (A_{ch}/A_{is}) over variable x (W_{ch}/W_{is}) where W_{ch} = cholesterol weight; W_{is} = internal standard weight; A_{ch} = cholesterol peak area; and A_{is} = internal standard peak area. The equation showed values of $a = -0.0681$, $b = 1.3957$ and $r^2 = 0.9992$ (coefficient of determination). This last value indicates excellent linearity. From this curve, we also calculated the RRF of cholesterol by using the expression $RRF = (W_{ch} \cdot A_{is})/(A_{ch} \cdot W_{is})$. The mean value (n = 28) of the RRF for cholesterol was 0.7764 [coefficient of variation (CV) = 8.17%].

Precision of the method. The intralaboratory precision was determined from data of ten repetitions of cholesterol determinations on the same fresh egg sample. The analyses were carried out by the same analyst, in the same laboratory, on successive days by using the same material and reagents.

Results of the ten determinations, as well as the mean value and variability of the cholesterol determination, are shown in Table 1. These results indicate a good precision for our method because the CV is low (1.39%).

Determination of the recovery of cholesterol. The same egg sample used in the precision determination was used in the recovery determination. Three different amounts of the cholesterol standard (2.4, 3 and 3.6 mg) were added to three 0.6-g fractions of the egg sample. We chose these three levels of addition because cholesterol contents usually reported in eggs are between 400 and 600 mg/100 g. So, the most probable amount of cholesterol present in the 0.6 g of sample would be between 2.4 and 3.6 mg.

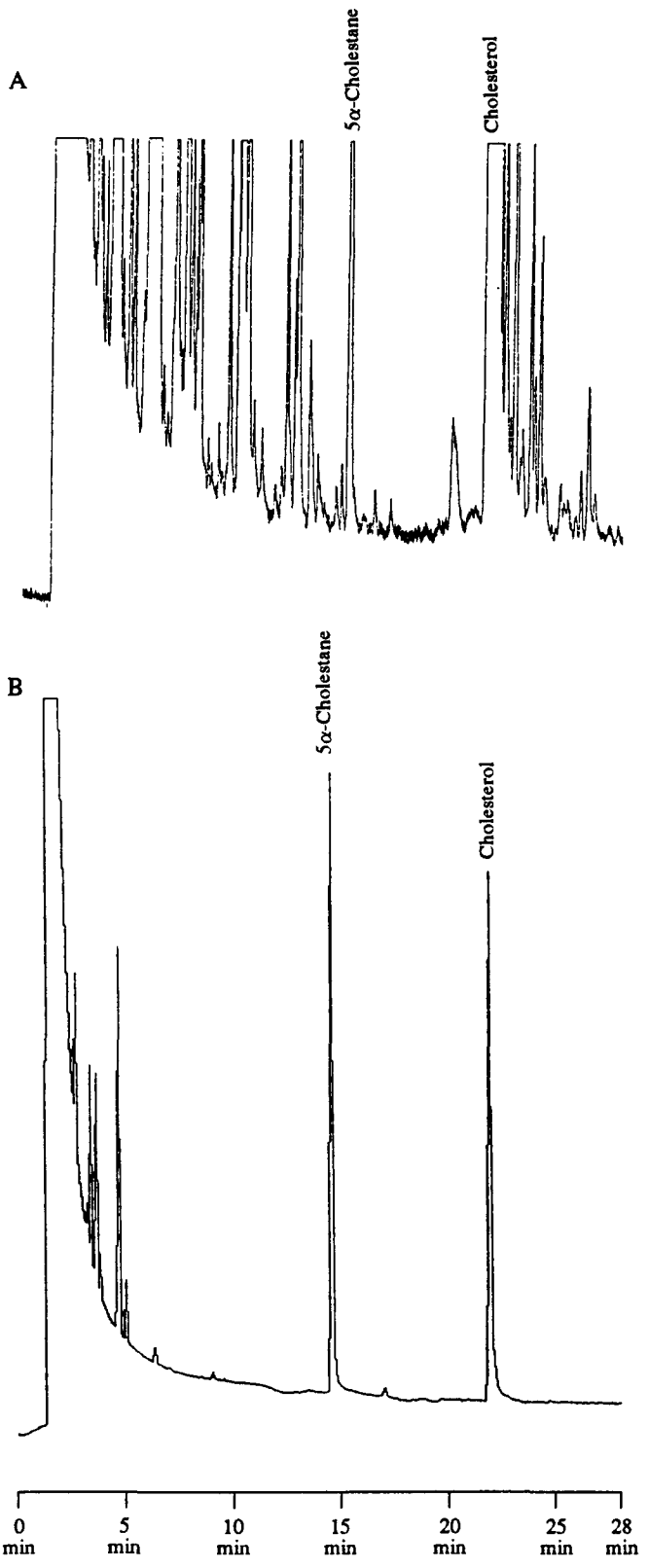

FIG. 1. Gas chromatogram of the nonsaponifiables of eggs after trimethylsilylation: (A) without dilution; (B) diluted.

TABLE 1

Precision of the Method for the Determination of Cholesterol[a]

Repetition	mg/100 g egg
1	390.2
2	379.9
3	380.4
4	381.6
5	391.8
6	389.8
7	383.3
8	384.4
9	376.6
10	390.6
X =	384.9
CV(%) =	1.4

[a]CV, coefficient of variation.

TABLE 2

Recovery of Cholesterol at Three Levels of Addition

Level of addition	Recovery (%)
2.4	60.8
2.4	70.3
2.4	67.5
2.4	64.8
2.4	69.5
$X_{2.4}$ =	66.6
CV(%) =	5.8
3	67.6
3	66.3
3	54.6
3	62.8
3	59.8
X_3 =	62.2
CV(%) =	8.4
3.6	61.3
3.6	60.7
3.6	62.6
3.6	65.2
3.6	62.1
$X_{3.6}$ =	62.4
CV(%) =	2.8
X_G[a] =	63.7
CV(%) =	6.5

[a]Global mean (n = 15). See Table 1 for abbreviation.

Quintuplicate determinations for every level of addition were performed, and the cholesterol recoveries obtained are shown in Table 2. There are differences between mean recoveries of cholesterol at the three levels of addition, but the analysis of variance for independent groups applied to these results indicates that the differences are not statistically significant. So, we calculated the global mean of all recovery data, which was used for the calculation of the cholesterol content in all samples. We emphasize that we prefer to work with a higher selectivity and precision, despite the lower percentage of recovery, because in this way the accuracy of the determination is clearly improved.

Cholesterol content in egg samples. Table 3 shows the results of the cholesterol determination in the egg samples analyzed. The ten first samples correspond to fresh eggs and the rest to frozen eggs. These cholesterol contents

TABLE 3

Contents of Cholesterol in the Egg Expressed as mg per 100 g of Edible Fraction[a]

Sample	Cholesterol
1	385
2	404
3	383
4	415
5	388
6	380
7	423
8	372
9	388
10	378
11	391
12	388
13	399
14	399
15	390
16	394
X	392
CV(%) =	3.4

[a]See Table 1 for abbreviation.

(mean value, 392 mg/100 g of edible portion) are lower than most of the data reported in food composition tables, although this level of content agrees with some of the results recently published (18,32). This is related to the fact, discussed above, that colorimetric methods present less selectivity and give higher cholesterol contents than chromatographic methods (18,19,30). All calculations were made by taking into account the recovery of the IS (65.8%, CV = 3.1%) and the recovery of cholesterol (63.7, CV = 6.5).

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