J. Korean Soc. Food Nutr.
23(6), 1032 ~ 1037(1994)

Culture Tube Method for the Determination of Total Cholesterol in Egg Yolk Lipid

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Abstract

A simple, reproducible, and accurate enzymatic method using a cholesterol assay kit was developed to quantify total cholesterol content in egg yolk. Total egg yolk lipid was extracted with hexane : isopropanol (3 : 2, v/v) mixture. Samples containing various amount of the total lipid(0~3mg) in optically identical culture tubes were reacted for 10min in a water bath(37° C) with the enzyme solution (5ml) from the cholesterol assay kit. Cholesterol content of the reaction mixtures in culture tubes was spectrophotometrically determined by two different ways : (1) using the culture tube as a cuvette (designate culture tube method ; CTM) and (2) the quartz cuvette containing the reaction mixture transferred from the culture tube (designate standard cuvette method, SCM). CTM revealed lower cholesterol content in 0.1~1.0mg lipid sample range than SCM did, but not significant. For more than 2.0mg lipid sample, CTM gave significantly ($p < 0.01$) lower cholesterol content relative to that by SCM, suggesting that SCM give a false positive result from the sample containing more than 2mg lipid due to the interference of absorbance by lipid dispersed in the reaction solution. Cholesterol content of less than 1.0mg lipid sample by CTM was proportional to the amount of lipid used, but its linear relationship was not seen in more than 2mg lipid sample. Thus, to determine the appropriate lipid amount for CTM, cholesterol concentrations (μg/mg) were plotted against total lipid amounts (mg) analyzed. A constant level(41μg/mg) of cholesterol concentration was observed from the sample containing 0.1~1mg lipid, after which the cholesterol level was dropped to less than 41μg/mg. Cholesterol concentration in egg yolk samples quantified by CTM was in accordance with that by GC method. These results suggest that CTM is an useful method for the quantification of cholesterol in egg yolk lipid and other lipids as well

Key words : cholesterol, egg yolk lipid, cholesterol assay kit

INTRODUCTION

Cholesterol is an important substance for the maintenance of integrity of cell membrane and for the precursor of steroid hormones as well as bile acids[1]. Contrast to these biological significances, cholesterol has detrimental effects on human chronic diseases like coronary heart disease and cancer[2,3].

As the major source of dietary cholesterol, cholesterol content of chicken eggs has recently received far more attention than before from farmers, medical professionals, and researchers[4-6]. Lots of methods have been developed to estate the cholesterol content in plasma, but few studies have been reported to apply these methods on the measurement of cholesterol content in egg yolk[6-8].

Currently, GC, HPLC, and enzymatic methods were found to be more accurate for the quantification of cholesterol in egg yolk with cold saponification of egg yolk lipid than other methods[9,10]. Somehow, in these methods, the procedure for the cold saponification of egg yolk lipid and the extraction of cholesterol and, moreover, analysis of cholesterol by GC or HPLC method are time consuming. Hence, currently, many researchers are enjoying the enzymatic method using a commercially available cholesterol assay kit for the quantification of cholesterol in egg yolk lipid.

Cholesterol assay kit is supplied for the determination of cholesterol content in sera[11], not for cholesterol in the egg yolk lipid. Thus, cares should be paid when applied this assay kit on the egg yolk cholesterol assay. When using this enzymatic method, we

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understood that lipid in the reaction mixture are interfering the absorbance of the reaction mixture at 540nm, resulting in difficulty in the duplication of the results.

To overcome these shortcomes, present study elucidates a simple, reproducible, and accurate spectrophotometric culture tube method, using a commercially available cholesterol assay kit, for the quantification of cholesterol in egg yolk lipid samples. The results from the enzymatic assay were compared to those from GC method to assure the accuracy.

MATERIALS AND METHODS

Materials

Cholesterol assay kit was purchased from Young yon Chemical Co., Japan. Hexane and isopropanol (all HPLC grade) were obtained from Mallinckrodt Specialty Chemical Co., (Paris, KY, USA). Culture tubes (10 × 100mm) were purchased from Corning Glass, USA. All other reagents were the reagent grade. Eggs layed from hens (160~170days of age) which were fed NRC normal diet (Table 1) were obtained from the Department of Animal Science, Gyeongsang National University, Chinju, Korea. Absorbance was recorded with Spectronic spectrophotometer (Milbon Roy Co., USA).

Table 1. Composition of experimental diet for the laying hens[1]

Ingredient	Composition(%)
Yellow corn	45.0
Wheat	12.0
Wheat bran	10.0
Rice bran	8.0
Soybean oil meal	9.0
Fish meal	7.0
Fat[2]	3.0
Bone meal	1.0
CaO	3.8
MgO	0.5
Vitamin mix	0.3
Salt	0.2
Brown seaweed[3]	0.2

[1] Diet contains amino acids and minerals to meet National Research Council specifications for laying hen's diet. Diet contains 2,928kcal/kg and vitamins (A, 6,839IU ; D, 5,512IU ; E, 40IU ; K, 0.75mg ; riboflavin, 8.6mg ; panthotenic acid, 19.5mg ; niacin, 8.8mg ; and B12, 0.02mg) per kg

[2] Fat source was soybean oil

[3] Brown seaweed was powdered after washing and drying

Total lipid extraction

Total lipid of egg yolk was extracted with slightly modified method of hexane : isopropanol (3 : 2, v/v) mixture[12]. Egg yolk (5g) dissolved in the hexane : isopropanol mixture (30ml) in a centrifuge test tube (40ml volume, teflon) was homogenized for one min with Homogenizer (Omni Instrument, USA) at a speed five position and then centrifuged at 4,000 rpm for 5min. The residue was reextracted with the solvent mixture (20ml × 2) and the supernatant (solvent layer) was combined with the previous supernatant. The combined supernatant was washed with 0.47M sodium sulfate solution (30ml × 3). Organic solvent fraction dried over sodium sulfate anhydrous was rotoevaporated (< 40° C) to get total lipid, which was weighed and dissolved in 2ml hexane (stock solution) and kept at 20° C until use.

Total cholesterol assay

Cholesterol assay kit method

Preparation of optically identical culture tubes

Glass culture tubes (100mm × 10mm, i.d.) with less than 0.001 absorbance unit difference at 540nm against the standard quartz cuvette (100mm × 10mm, i.d.) were prepared. These were used as vessels for the enzymatic reaction and directly used as cuvettes to measure absorbance of the reaction solution at 540nm.

Total cholesterol assay by cholesterol assay kit

Aliquot amount of total lipid solution containing 0.1, 0.2, 0.5, 1.0, 2.0, and 3.0mg lipid was taken in the glass culture tubes from the stock solution and dried under nitrogen. The lipid sample was reacted in a water bath (37° C, 5min) with the enzyme solution (5ml) containing cholesterol oxidase (17.5unit), cholesterol esterase (9unit), peroxidase (50unit), 4-aminoantipyridine (6.4mg) and phenol (198mg). Absorbance of the reaction mixture in the glass culture tube was measured at 540nm by two different ways : using the culture tube directly as the cuvette (designate culture tube method, CTM) and secondly using a quartz cuvette containing reaction mixutre transferred from the culture cuvette measured the absorbance (designate standard cuvette method, SCM). As results, reac-

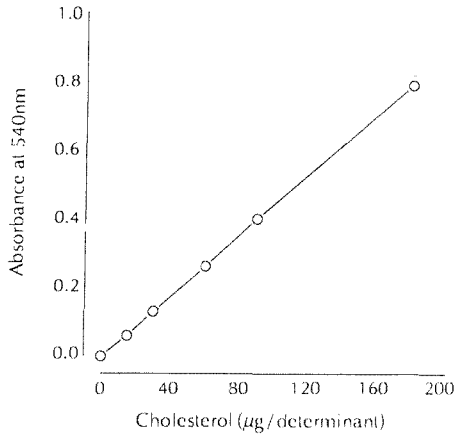

Fig. 1. External standard curve for the quantification of cholesterol in egg yolk lipid. Cholesterol standard and all other reagents including enzymes were provided by the supplier described in Materials.

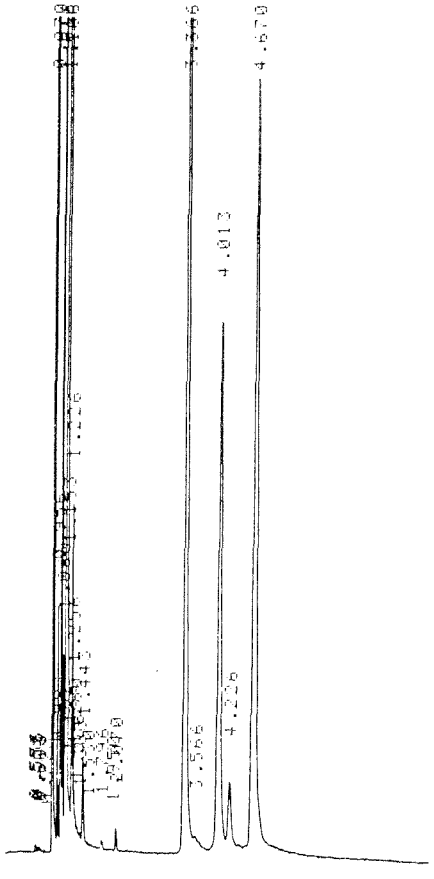

Fig. 2. GC chromatogram of cholesterol from egg yolk lipid. Condition was detailed in Method. Peaks with retention time of 3.37 and 4.67 are cholesterol and β-sitosterol, respectively.

reaction mixture was disturbed in SCM, but not in CTM. Total cholesterol content was determined by the external standard method (Fig. 1).

GC method

Cold saponification of the total lipid

Total lipid extract (approximately 100mg) from egg yolk containing 10mg β-sitosterol as an internal standard (IS) was saponified (37° C, 3hrs) with 8.6M KOH solution (0.12ml) plus absolute ethanol (4ml). Unsaponifiable materials was extracted with hexane (2ml × 3) in the presence of water (1ml) by shaking for one min and separated by centrifuging at 4,000rpm for 5min. The organic layer dried over sodium sulfate anhydrous was used for the analysis of cholesterol by capillary GC.

GC conditions

Hewlett Packard 5890 series II GC was equipped with FID and capillary column (Ultra 1, 60m × 0.32 mm, i.d.). Oven temperature was the isotherm at 270° C. Both injector and detector temperatures were 300° C. Nitrogen was used at flow rate 2ml/min as a carrier gas. Cholesterol and β-sitosterol (IS) were eluted at 5min and 10min, respectively (Fig. 2).

RESULTS

Development of CTM

Fig. 3 shows cholesterol content in the egg yolk lipid determined by CTM and these results were compared to those by SCM. No significant difference in cholesterol content measured by CTM and SCM was observed from <0.5mg lipid sample, but the significant difference ($p<0.05$ by t-test) was seen in >1.0mg lipids. Cholesterol content in 1mg lipid sample quantified by CTM and SCM was appeared to be 39μg and 44μg, respectively, indicating that CTM gave 11% less cholesterol for the same sample as compared to that from SCM. Similarly, CTM revealed 42% less cholesterol in 3mg lipid sample than SCM did. These results suggest that CTM provided significantly ($p<0.05$) lower cholesterol content in the range of 2.0~3.0mg lipid sample than SCM did.

Accuracy of CTM was further supported by plotting the cholesterol concentration (μg/mg lipid) agai-

nst various amount of the lipid sample used (Fig. 4). Cholesterol concentration measured by CTM was lower in tested range of lipid sample than that by SCM. Cholesterol concentration in 0.1~1.0mg lipid sample determined by CTM was found to be constant as about 41μg/mg lipid. Meanwhile it was found to be 45~43μg/mg in 0.1~1.0mg lipid sample by SCM. The cholesterol concentration determined by the two methods was not significantly different in the range of 0.1~1.0mg lipid, but CTM revealed significantly lower cholesterol concentration in >2mg lipid than SCM did.

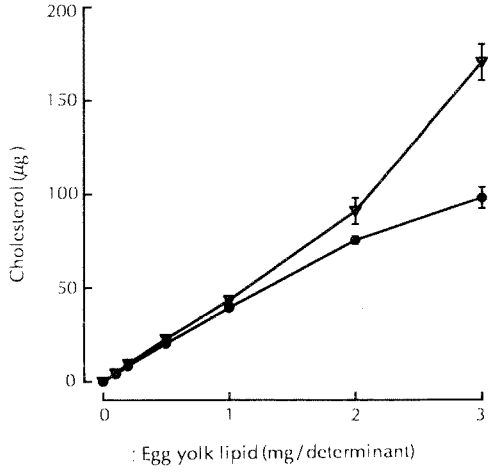

Fig. 3. Cholesterol content in the egg yolk lipid determined by CTM using the cholesterol assay kit. Cholesterol content represented as closed circles and closed triangles are determined by CTM and SCM, respectively.

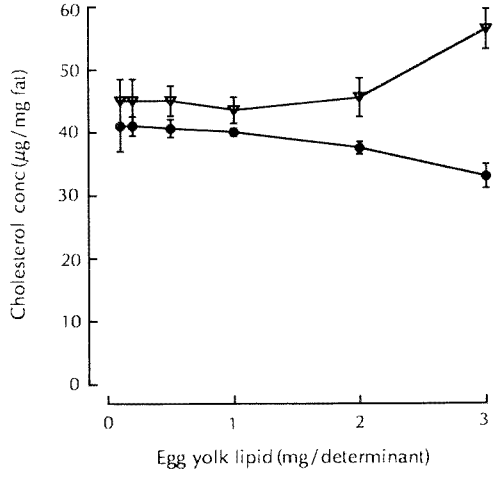

Fig. 4. Relationship of cholesterol concentration (μg/mg) and the amount of egg yolk lipid analyzed by CTM (closed circles) or SCM (closed triangles) using the cholesterol assay kit.

Quantification of cholesterol by CTM

CTM was used to quantify cholesterol content of egg yolk lipid samples and the result was compared to that of GC analysis (Table 2). No difference in cholesterol content determined by CTM and GC was observed. Given these results, CTM might be as accurate as GC to measure cholesterol content in egg yolk lipid.

DISCUSSION

Cholesterol assay kit provided for the determination of cholesterol content in serum containe enzymes responsible for the hydrolysis of cholesterol ester and the oxidation of free cholesterol, and reagents reacting with the oxidized cholesterol to develop the color with maximum absorbance at 540nm. When one uses this assay kit for cholesterol analysis in lipid, i.e., egg yolk lipid, which conditions are not similar to blood sera, disadvantages encountered are poor solubility of the lipid and the limitation of sample amount to be analyzed. Enzymes oxidize cholesterol, but not triglycerides, the fact that lipid is floated on the top and/or dispersed in the reaction mixture. Given this phenomenon, false positive results in absorbance at 540nm are derived due to interfering the light path through the cuvette by lipid dispersed in

Table 2. Comparison of the egg yolk cholesterol content (mg/g) assayed by CTM and GC methods

Sample[1]	CTM[2]	GC method[3]
1	3.75 (0.34)[4]	3.80 (0.50)
2	4.30 (0.53)	4.28 (0.41)
3	3.56 (0.23)	3.48 (0.32)
4	3.57 (0.20)	3.45 (0.44)
5	4.02 (0.21)	3.98 (0.60)

[1] Each sample containing 5 eggs was collected from the hen (160~170days of age) fed the diet described in Table 1 for 2 weeks. Egg yolk lipid was extracted from egg yolk by hexane : isopropanol (3 : 2, v/v) mixture. Total cholesterol content in egg yolk lipid was analyzed by CTM and GC method
[2] CTM was described in Method
[3] GC method was described in Method
[4] Parenthesis represents standard error mean of 4 measurements

the reaction solution. CTM developed for the quantification of total cholesterol in egg yolk lipid could overcome this spectrophotometrical disadvantage from which cholesterol assay kit was directly used to evaluate cholesterol content in egg yolk lipid.

Under the test condition, CTM consisted of the total lipids from egg yolk (1mg), optically identical culture tubes which are measured at 540nm, and enzyme solution (5ml) from the cholesterol assay kit. Advantage of this method is that the reaction mixture was not disturbed, when the absorbance was measured, due to the direct use of the culture tube as a cuvette. CTM gave similar cholesterol content to that measured by GC method. It is note worth that, in CTM, as much as 1mg lipid sample, not exceed 2mg, could be used to get reliable results without any interference by lipid. CTM is simple, reproducible, and accurate, as compared to SCM and/or GC. An expert can analyze more than 100 samples per day.

CTM is comparable to the standard enzyme method in easiness and acuracy of the assay. In the enzyme method, which is commonly used in laboratory for the assay of egg yolk cholesterol, an enzyme solution, which is freshly preparing by mixing an individual enzyme solution of peroxidase, cholesterol esterase and cholesterol oxidase, reacted in the presence of detergent, i.e., Triton X-100, with egg yolk lipid for 10min at 37° C[13]. The detergent used might inhibit the enzymatic reaction with substrates and also might interfere the measurement of color developed. Only a cuvette was used for the measurement of color intensity, resulting in the disturbance of the reaction mixture and an extensive time for the assay. In contrast to enzyme method, CTM used a ready-made reaction mixture (cholesterol assay kit) and 40 culture tube cuvettes for the measurement of absorbance, and did not require any detergents.

This method might be applied for the determination of cholesterol in any other lipid derived from food stuffs. In this case, one should determine an appropriate amount of enzyme solution to be used before initiation of the assay since the enzyme solution is dependent upon cholesterol content.

ACKNOWLWDGMENTS

We thank Kyeong N. Bahn, Sook J. Park, and Jae I. Byun for their technical assistance. This work was supported by the research grant 921-1500-103-2 from KOSEF.

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(Received October 13, 1994))

Culture Tube 방법에 의한 난황중의 Cholesterol 정량

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요 약

달걀 중의 cholesterol 정량에는 GC, HPLC 및 효소 방법이 이용되고 있지만 GC나 HPLC 방법은 시간과 장비의 문제점이 있어 간편한 효소법이 가장 많이 사용된다. 그러나 효소법을 난황지방에 직접 사용할 경우 효소에 의해 분해되지 못한 지방이 비색 측정을 방해하여 실제 보다 과량 검출되거나 반복간의 차이를 유발하여 실재의 함량을 측정하는데 큰 오류를 범할 수 있다. 따라서 본 연구에서는 효소법의 이와 같은 단점을 보안하고 실제로 실험실에서 쉽게 사용할 수 있도록 culture tube method (CTM)를 개발하였다. 효소의 제재로서는 시중에서 구입할 수 있는 cholesterol assay kit를 사용하였다. 난황에서 분리한 지방 (0.1, 0.2, 0.5, 1.0, 2.0, 3.0mg)을 culture test tube (540nm에서 흡광도의 차이가 0.001 이하)에 각각 넣고 여기에 효소액 5ml를 가하여 37°C 항온조에서 10분간 반응시킨 후 2가지 방법으로 540nm에서 흡광도를 측정하였다. 첫째는 culture tube를 cuvette으로 사용하여 반응물이 흔들리지 않게 spectrophotometer에서 흡광도를 측정하였고 (Culture tube method, CTM) 다음은 이것을 다시 정상 cuvette에 옮겨 흡광도를 측정하였다 (standard cuvette method, SCM : 이 경우 반응물이 혼합됨). 이 결과 난황지방 1.0mg 까지는 CTM에 의해 측정한 cholesterol 함량은 사용된 지방 함량에 비례하였지만 그 이상의 지방 함량에서는 비례하지 않았다. 그리고 CTM으로 측정된 cholestrol 함량은 SCM에 의한 결과 보다 낮은 수치를 보여 1.0mg에서는 11%, 3.0mg에서는 42% 차이 ($p<0.01$)를 보였다. CTM에 이용 가능한 난황지방 함량을 구하기 위해 CTM에 의해 정량된 cholesterol 함량을 사용된 지방 함량으로 나누어 본 결과, 사용된 지방의 함량 1.0mg 까지는 mg당 cholesterol 함량이 약 41μg/ mg으로 일정하였으나 2mg 이상인 경우는 그 이하로 떨어졌다. 이 결과로 미루어 상기 조건하에서는 사용 가능한 지방량의 한계는 1mg으로 나타났다. CTM으로 측정한 실제 달걀 중의 cholesterol 함량은 GC법으로 측정한 cholesterol 함량과 일치하였다. 이상의 결과로서 난황에서 추출한 지방에 함유된 cholesterol의 함량을 cholesterol assay kit를 사용하여 CTM법으로 측정하기 위하여서는 지방 함량 0.5~1.0mg에 효소액 5ml를 사용하는 것이 가장 바람직하다. CTM를 이용하면 숙련된 사람일 경우 적어도 하루에 50~100개의 시료를 분석할 수 있다.