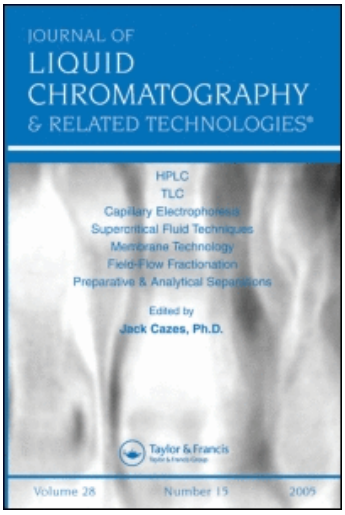

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DETERMINATION OF CHOLESTEROL IN EGG YOLK BY HIGH PERFORMANCE TLC WITH DENSITOMETRY

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ABSTRACT

A simple quantitative TLC determination of cholesterol in egg yolk is described. Cholesterol in the yolk is extracted with chloroform-methanol (2:1) and the extract filtered through glass wool. After dilution to a known volume, samples and standards are developed on a high performance preadsorbent silica gel layer, cholesterol is detected with cupric acetate-H_3PO_4 reagent, and zones are scanned and compared. Precision of the method, evaluated by replicate analyses, was 2.2%, and recovery from a sample spiked at a concentration level of 14.3 mg/g was 104%. Analysis of 20 eggs gave cholesterol values ranging from 9.7-26.2 mg/g, and the method was also applied to butter and cream samples. Because of the selectivity of the TLC method, lower results were obtained compared to a standard colorimetric assay. Fractions representing sterols, diacylglycerols, triacylglycerols, stearyl esters, and hydrocarbons were identified from egg yolk, and the sterol fraction was found to contain only cholesterol by argentation- and RP-TLC and capillary GC.

INTRODUCTION

The recent increasing concern about the health implications of dietary cholesterol and the advent of Federal voluntary food labeling regulations (1) have led to the need for improved methods for determining cholesterol in foods. The official AOAC method (2) for cholesterol in egg and egg products, based on titration with sodium thiosulfate, is cumbersome and time consuming and lacks specificity. The most widely used methods are spectrometric (3,4), but these, too, are nonspecific, detecting compounds other than cholesterol and other sterols, such as cholesteryl esters. Gas chromatography (GC) (5-8) and HPLC (9-12) have been employed recently with increasing frequency. These techniques are more specific, sensitive, and reliable, but have the disadvantage of generally requiring prior derivatization of cholesterol. The determination of cholesterol in egg yolk by an enzymatic/spectrometric technique following extraction and saponification of the lipid extract has also been reported (13).

Quantitative densitometry has been widely used for the determination of cholesterol and other lipids in biological samples such as blood, urine, bile, and semen (14-16), but analysis of egg yolk and most other food samples for cholesterol has not been reported. Wood and Bitman used quantitative TLC to measure lipid classes in hen plasma and egg yolk, but they determined cholesterol by GC of the TMS-derivative (17,18). In this paper we report the qualitative pattern of neutral lipids in chicken egg yolk and describe a

rapid and simple HPTLC method for quantification of cholesterol. The accuracy and precision of the method were evaluated, and it was compared to a standard spectrometric assay. The method was used to survey the cholesterol content of a number of egg yolks and, to demonstrate its applicability, the method was used, as well, for the analysis of light cream and butter samples. The sterol fraction of egg yolk was isolated by preparative silica gel TLC and shown to contain predominantly cholesterol by reversed phase (RP) TLC on C-18 layers, argentation-TLC, and capillary GC. Finally, the separation of a series of sterols by RP- and argentation-TLC was demonstrated.

EXPERIMENTAL

Standards and Standard Solutions

Cholesterol standard (99.99% purity) was purchased from Starks Associates. A stock solution was prepared at a concentration of 1.00 mg/ml in chloroform, and TLC standard was prepared by a 10-fold dilution with chloroform (100 ng/µl). Mixed lipid standards 18-1A and 18-4A were purchased from Nu-Chek Prep, Inc.; 18-1A contains monolein, diolein, triolein, and methyl oleate; 18-4A includes cholesterol, oleic acid, triolein, methyl oleate, and cholesteryl oleate. Other sterol standards were obtained from Sigma Chemical Company and Supelco, with the highest purity available. Standard solutions of sterols at appropriate concentrations were prepared in chloroform.

TLC Procedures

Baker-Flex IB2 20 x 20 cm silica gel sheets were used for qualitative analysis of lipid classes and argentation-TLC of sterols; Whatman LHPKDF 20 x 10 cm channeled high performance silica gel plates with preadsorbent spotting area for quantitative densitometry; Whatman PKIF 20 x 20 cm plates with 0.5 mm silica gel layer thickness for preparative TLC; and Whatman LKC18F 20 x 20 cm preadsorbent bonded silica gel plates for reversed phase separations of sterols. All layers were cleaned by development with methanol-methylene chloride (1:1) and allowed to air dry before use.

Initial zones were applied using a Drummond 10 µl microdispenser. For quantitative TLC, 100 to 1000 ng of cholesterol standard (1-10 µl of the 100 ng/µl solution) and duplicate 5 µl portions of the final sample solutions were spotted.

Layers were developed in saturated, paper-lined glass TLC tanks and allowed to air dry. For quantification, lipids were detected on HP silica gel with cupric acetate-H_3PO_4 reagent (14). For qualitative and preparative TLC on silica gel and for reversed phase TLC, detection was made using phosphomolybdic acid reagent (14). Sulfuric acid charring was used to detect sterols on Ag^+-impregnated silica sheets.

Lipid zones were scanned using a Kontes Model 800 densitometer equipped with a Hewlett-Packard Model 3992A integrator/recorder. Parameters and general procedures for scanning and sample quantification were described earlier (19).

The sterol fraction from 3 ml of egg yolk (representing ca. 12 mg of lipid) was isolated by preparative TLC following the procedures described by Fried and Sherma (20). A 1 ml chloroform solution of the sample was streaked on the preparative layer using a 100 µl micropipet, and the sample was focused (concentrated) by a short (2 cm) development with methanol. The methanol was evaporated and the plate developed with the Skipski et al. (21) dual-solvent system, which consists of a 14 cm run with isopropyl etheracetic acid (96:4) followed by a 19 cm run in the same direction with petroleum ether (60-70°C)-diethyl ether-acetic acid (90:10:1). The separated sterol band was scraped and packed into a microcolumn (20), and the sterols were eluted with acetone.

The preparatively isolated sterol band and a similarly-treated cholesterol standard were compared by capillary GC using a 15 meter fused silica column coated with a 0.25 µm film of bonded methyl silicone and a temperature program (190°C-260°C) that was known to resolve a large number of sterols. The sterol fraction was also chromatographed on C-18 RP and silver-impregnated silica gel layers. Argentation TLC was performed as described earlier (21), except that the Baker-Flex layer was impregnated with 2.5% ethanolic $AgNO_3$ rather than 5%.

Sample Preparation Procedures

Chicken's egg samples to be quantitatively analyzed for cholesterol were purchased in a local supermarket and stored at 10°C for no more than one week. Eggs were broken and the yolk

was separated manually from the white. The yolk was homogenized in a 50 ml beaker with a stirring rod, and a ca. 70 mg sample was removed with a disposable Pasteur pipet into a tared 8 ml vial. Butter (150 mg) and light cream (1.5 g) samples were weighed directly into vials. Two ml of chloroform-methanol (2:1) was added to the egg and butter samples and 10 ml to the cream. The vial was hand-shaken and the sample then filtered through a disposable pipet plugged with glass wool (illustrated on page 169 of Reference 20). The vial was rinsed three times with the solvent and the rinsings passed through the glass wool. The sample was evaporated to dryness under nitrogen and then diluted with exactly 10 ml of solvent added by pipet. Duplicate 5 µl aliquots of this solution were applied with bracketing standards for TLC.

RESULTS AND DISCUSSION

The lipids present in egg yolk were identified by development of extract and two standards containing representatives of all neutral lipid classes (18-1A and 18-4A) on a Baker silica gel sheet with the Skipski et al. dual-solvent system, described in the experimental section. The neutral lipid fractions, identified by PMA reagent based on their migration relative to standards, were sterols (cholesterol), R_F 0.40; diacylglycerols (diolein), R_F 0.43; triacylglycerols (triolein), R_F 0.77; stearyl esters (cholesterol oleate), R_F 0.85; and hydrocarbons, R_F 0.95. A heavy spot at the origin presumably contained phospholipids, which were reported (22)

to constitute about 32% of the lipid content of hen's egg yolk. The relative concentrations of the neutral lipids were triacylglycerols > sterols > diacylglycerylols > stearyl esters.

For quantitative determination, chromatograms were developed on preadsorbent silica gel plates with chloroform-ethyl acetate (94:6). Cupric acetate-H_3PO_4 dip reagent detected cholesterol zones as tight brown streaks on a faintly blue background with an R_F value of 0.47. Plates were scanned immediately because some fading began to occur about one hour after detection. The other neutral lipids and hydrocarbons were in a mixed zone at R_F 0.9 and phospholipids remained in the preadsorbent with this mobile phase.

Figure 1 shows typical scans for a series of cholesterol standards in the ca. 100-1000 ng concentration range. Calibration plots (peak area vs ng spotted) generally had linearity correlation coefficient (R) values of 0.98 or greater. Standards were always spotted with samples to provide a unique calibration plot for each plate, thereby correcting for variations in slope and intercept values. Scans of duplicate sample aliquots are also shown in Figure 1.

The sterol fraction from egg yolk was separated and recovered by preparative silica gel TLC. After scraping and elution, the solution was analyzed by reversed phase- and argentation-TLC and by capillary gas chromatography under conditions optimized for separation of the maximum number of sterol standards. Comparison of the TL and gas chromatograms with those of a cholesterol standard that

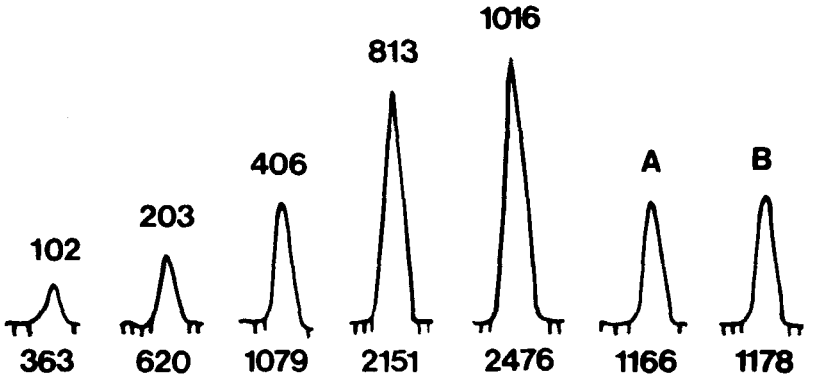

Figure 1. Densitometer scans of 102-1016 ng cholesterol standards and duplicate 5 µl aliquots of an egg yolk extract, made at an integrator attenuation setting of X8. The standard areas (shown below the peaks) had a linearity correlation (R) of 0.998, and the sample peak areas represent 12.8 (A) and 13.0 (B) mg of cholesterol per gram of yolk.

was processed identically by preparative silica gel TLC showed that cholesterol was the only sterol that was detected in the yolk extract. These results, which were similar to those of Singh et al. (23) for hen's egg yolk, proved that the sterol zone separated by silica gel TLC could be reliably quantified using a pure cholesterol standard. Table 1 shows R_F values for a variety of sterols separated by C-18 RP- and argentation-TLC. In both systems, tight, circular zones were formed. Resolution by argentation-TLC was somewhat better and zones with different colors were produced, which facilitates identification of the sterols.

To determine if there were variations in cholesterol content in different locations, samples were taken from the center and opposite edges of a single, intact yolk. The values obtained were 25.8, 24.2 and 26.2 mg cholesterol/g (relative standard deviation

TABLE 1

R_F VALUES OF STEROLS ON C-18 RP AND Ag^+-IMPREGNATED LAYERS

Sterol	RP[a]	Ag[b]	
cholesterol	0.36	0.53	(G)
ergosterol	0.41	0.49	(P)
desmosterol	0.40	0.53	(G)
stigmasterol	0.36	0.52	(P)
coprostanol	0.39	0.60	(B)
cholestanol	0.32	0.51	(B)
lanosterol	0.46[c] 0.41	0.60	(B)
β-sitosterol	0.36	0.48	(P)
fucosterol	0.39	0.47	(G)

[a] C-18 bonded silica gel layer developed with acetonitrile-chloroform (40:35); all sterols were detected as blue spots by PMA.

[b] Silica gel sheet impregnated with 2.5% ethanolic $AgNO_3$ and developed with chloroform-acetone (95:5). Zones were detected by charring with 50% aqueous H_2SO_4 to produce the colors shown in the parentheses: G = grey, P = purple, B = brown.

[c] The lanosterol standard had a stated purity of 65%; two zones were separated in this system.

3.4%), indicating an insignificant effect of the sampling site. To insure that results were not affected by this variable, however, yolks were homogenized before sampling for all subsequent analyses.

The precision of the method was evaluated by analyzing four different samples from a single homogenized yolk. The relative standard deviation of the replicate analyses was an excellent 2.2%.

The accuracy of the method was proven by a standard addition analysis. Two samples from the same yolk were taken, and one was fortified by addition of 1 ml of the 1 mg/ml cholesterol standard. The difference between the cholesterol concentrations found for the two samples divided by the amount added resulted in a recovery of 104%.

The TLC method was compared to the standard Pearson et al. (3) colorimetric method for cholesterol as modified by Bair and Marion (24) for hens' eggs. Four samples were taken from the same yolk, and two each were analyzed by TLC and colorimetry. The average values were 12.0 mg/g for TLC and 17.0 mg for colorimetry. The higher value was undoubtedly due to the nonspecificity of the colorimetric method, which is known to determine cholesteryl esters as well as free cholesterol ("total cholesterol") (3).

The cholesterol contents of 20 egg yolk samples determined by the TLC method are presented in Table 2. In the case of some eggs, multiple analyses were performed (e.g., two replicates for egg number 2 and three for number 3). The values range from 9.7 to 26.2 mg/g, which is similar to the wide range of values reported in the literature for yolk cholesterol in eggs from various avian species (24). The variation in cholesterol levels for different

TABLE 2

CHOLESTEROL CONTENT (MG/G YOLK) OF EGG YOLK, BUTTER, AND CREAM AS MEASURED BY THE QUANTITATIVE TLC METHOD

Sample Number[a]	Cholesterol
1	19.8
2	13.9
2	14.0
3	25.8
3	24.2
3	26.2
4	9.72
5	11.8
6	10.7
7	9.84
8	10.9
9	19.6
10	18.6
11	17.5
12	14.4
13	15.9
14	12.6
15	12.8
15	13.4
15	13.6
15	13.3
16	13.4
17	11.4
17	12.6
18	14.4
18B	12.0
18F	14.3
19	2.68
19	2.59
20	0.485
20	0.506

[a] Samples 1-17 were fresh chicken egg yolk; samples 18, 18B, and 18F were fresh, boiled, and frozen yolk, respectively; sample 19 was butter; sample 20 was light cream.

eggs is most likely due to differences in feeding, environment, and other factors pertaining to the care and maintenance of the chickens. One yolk sample (number 18, Table 2) was divided into three parts; one part was frozen at -20°C overnight, another was boiled in water inside a vial for 30 minutes, and one was stored in a refrigerator. No significant effect on the cholesterol level was caused by boiling or freezing.

The method, essentially unchanged, was also applied to the analysis of one sample each of light cream and butter to demonstrate its versatility. The 2.64 mg/g average obtained for butter matched closely the value on the packaging. The cream averaged 0.495 mg/g, which converts to 0.0495%, the label value in this case being 0.064%. Further work to explain this discrepancy for the cream was not carried out; our purpose was only to demonstrate the applicability of the isolation and determinative methods for food types other than egg.

The quantitative TLC procedure is suitable for routine analysis of cholesterol in egg yolk, being relatively simple, accurate, precise, and rapid on a per-sample basis. The data in Table 1 are a useful aid for identifying the sterols from foods, plants, and organisms that do not contain only cholesterol in the sterol band separated by silica gel TLC.

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