Development and validation of a high-performance thin layer chromatography method for the determination of cholesterol concentration

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Abstract
An accurate, sensitive, precise, reliable, and quick method for the determination of cholesterol content by high-performance thin layer chromatography is developed. In this method, aluminum-backed precoated silica gel 60 F254 plates were used as the stationary phase and the samples were sprayed with the help of CAMAG Sample Applicator Linomat 5. The chromatogram was developed with the mobile phase consisting of chloroform:methanol (9.5:0.5, v/v). The samples were detected using CAMAG Scanner 4 and evaluated using the method developed on winCATS software. Densitometric analysis of cholesterol was performed in absorbance mode at 200 nm. In this solvent system, cholesterol gave a compact spot with an Rf value of 0.63 ± 0.03. The linear regression analysis of data for the calibration curve showed good linearity over a concentration range of 2–7 μg/spot with a regression value of 0.99933 and standard deviation of 1.44%. The limit of detection and limit of quantification were found to be 100 ng/spot and 500 ng/spot, respectively. Using the developed method, the concentration of cholesterol in the saponified and unsaponified egg yolk sample was determined. This method was found to be reproducible and can even be used for samples containing complex matrices.

1. Introduction
Cholesterol is considered within the class of lipid molecules and is vital for normal body function. It is an essential structural component of animal cell membranes and is required to maintain proper membrane permeability and fluidity. Since the relationship between plasma cholesterol concentration and atherosclerosis was demonstrated in...
rabbits in 1913, the interest in evaluating the content (concentration) of cholesterol in foods has been driven by the awareness of the association between dietary cholesterol and human disease. As a result, cholesterol has become an important component of composition studies on various food products [1].

Research on the various biological functions of cholesterol and concerns over its pathophysiology are the driving force for the development and application of analytical methods for the determination of cholesterol concentration in various food products. Cholesterol (Fig. 1) is water insoluble, making its concentration determination difficult because of the presence of lipids. Complicated techniques, such as isotopic tracing and mass fragmentation, were originally developed for qualitative detection of cholesterol to study its biosynthetic pathways and metabolism in cells, although these techniques are also used in quantitative estimation. Various kinds of methods to determine the concentration of cholesterol have been reported, including colorimetric and spectrophotometric estimations [2–6], gas–liquid chromatography [7,8], high-performance liquid chromatography (HPLC) [9], and fluorometric and other methods [10,11]. Most of the reported protocols involve the use of complex reagents or complicated methods, and some of these are not applicable for samples containing complex matrices.

High-performance thin layer chromatography (HPTLC) is a sophisticated instrumental technique that utilizes the full capabilities of thin layer chromatography. The attractive features of HPTLC are as follows: low-cost analysis of samples, requires minimal sample clean up, and allows for a reduction in the number of sample preparation steps. TLC is an offline technique: the subsequent steps are relatively independent, allowing for parallel treatment of multiple samples during chromatography, derivatization, and detection. HPTLC plays an important role as a modern analytical tool, which is equally suitable for qualitative and quantitative analytical tasks. One of the most obvious features of this technique is that it allows for simultaneous analysis of multiple samples containing multicomponents, so that reference and test samples can be compared for identification. In addition to the visible chromatograms, peak data can also be obtained by densitometric evaluation of the chromatogram by using a TLC scanner, measuring the absorption and/or fluorescence of the substances on the plate and these densitometric data can be quantitatively evaluated [12]. Because lipoproteins are of high interest in the context of atherosclerosis research, many papers dedicated to the analysis of cholesterol and its derivatives by TLC are available and these are summarized in the review by Touchstone [13]. Most of the methods are based on mass spectrometry or postchromatographic derivatization by reagents for detection of cholesterol concentration.

The aim of the study was to develop and validate a simple HPTLC method for determination of cholesterol concentration. The analytical procedure was characterized to ensure its selectivity, accuracy, and precision for the analysis. Cholesterol content in an egg yolk sample was determined to validate the accuracy of this method.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals used in the experiments were of analytical grade. Cholesterol (95% pure; analytical standard) was purchased from Sigma Life Science. All the solvents and chemicals used were of analytical grade purchased from Merck (Mumbai, India). KOH (5.6%) in methanol was used as saponifying agent.

2.2. Instrumentation and conditions

Aluminum-backed HPTLC plates precoated with silica gel 60 F254 (10 cm × 10 cm) from Merck (Germany) were used as the stationary phase. The samples were sprayed on the HPTLC plate (spray-on technique) with the help of a sample applicator (Linomat 5; CAMAG) under nitrogen gas flow. The chromatogram was developed in 10 cm × 10 cm twin trough chambers (CAMAG). Densitometric analysis was carried out using CAMAG TLC Scanner 4, fitted with winCATS planar chromatography manager software (CAMAG, Muttenz, Switzerland, version 1.4.8.2012). The TLC plates were dried with a hair drier or in an oven.

2.3. Standard preparation

Cholesterol standard (0.1 mg/mL) was prepared by dissolving 1 mg cholesterol in 10 mL of methanol; this solution was then sonicated in Sonica Ultrasonic Cleaner (Spinco Biotech Pvt. Ltd., Mumbai, India) for 20 minutes to obtain a homogenized solution. The solution was stored at 4°C in dark.

2.3.1. Sample preparation

An average-sized white leghorn egg purchased from the local market was washed properly and then hard-boiled in distilled water. The egg yolk (14.95 g) was carefully separated from the hard-boiled egg (55.5 g) and rolled on a filter paper until complete removal of egg white. For the preparation of saponified sample, 1 g of the egg yolk was dispensed in 2 mL of ethanol to which 18 mL of alcoholic KOH was added and mixed in a conical flask. The sample was then sonicated at 60°C for 30 minutes in Sonica Ultrasonic Cleaner water bath (Spinco Biotech Pvt. Ltd.) for proper homogenization. The resultant mixture was then transferred into a separating funnel and extracted with 10 mL hexane with regular shaking.

Fig. 1 – Structure of cholesterol.
for 1 hour. To ensure complete extraction of cholesterol, the extraction process was repeated twice with 5 mL of hexane. The hexane layer was then pooled and evaporated to dryness. The hexane extract was then dissolved in 10 mL methanol and used as the working sample. For unsaponified sample preparation, 1 g egg yolk was dissolved in 20 mL of 90% methanol. The sample was then homogenized in the sonicator for 30 minutes at 60°C. The solution was then transferred into a separating funnel and extracted with hexane as described earlier. The prepared samples were stored at 4°C in dark.

2.4. Chromatographic conditions

Samples and standards were prepared in methanol and sprayed on the HPTLC plate using a sample applicator (Linomat 5). Prior to performing quantitative analysis, the mobile phase was optimized for better resolution by applying the trial-and-error method. The optimized solvent system was then used as the mobile phase for further analysis. Prior to sample application, the plates were washed in methanol and activated by drying in an oven. Spots of this sample were applied with a band length of 6 mm, with a distance of 7.8 mm between each track. Samples were sprayed at speed of 150 nL/second with nitrogen as the spray gas using an analytical syringe (CAMAG) of 100-μL capacity. The chromatogram was developed in CAMAG twin through chamber (10 cm × 10 cm) with 10 mL of the mobile phase consisting of chloroform:methanol (9.5:0.5, v/v). Application positions were at least 10 mm from the sides and 10 mm from the bottom of the plates. The mobile phase components were mixed prior to use and the development chamber was allowed to saturate with mobile phase vapor for 20 minutes prior to each run by placing filter paper moistened with the solvent system in the twin trough chamber. Development of the plate was carried out by the ascending technique to a migration distance of 70 mm. The plates were then dried on a hot plate. Detection and densitometric scanning were performed by CAMAG TLC Scanner 4 (Swiss made) in absorption mode at 200 nm with the slit dimension 5 mm × 0.3 mm. The sample track scanning speed was 20 mm/second and spot spectrum scanning speed was 100 nm/second. The entire process was performed under the control of winCATS planar chromatographic manager software (CAMAG, Muttenz, Switzerland, version 1.4.8.2012). All the analyses were carried out in laboratory at controlled room temperature (25 ± 3°C).

2.5. Method validation

The developed method was validated according to the International Conference on Harmonization (ICH) guidelines with respect to linearity and range, specificity, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ). According to the ICH guideline, validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for the intended analytical applications. The accuracy of an analytical procedure is the closeness of test results obtained by that procedure to the true value. The accuracy of an analytical procedure should be established across its range. The precision of an analytical procedure is defined as the degree of agreement among individual test results when the procedure is repeated for multiple samplings of a homogeneous mixture. The precision of an analytical procedure is usually expressed as the standard deviation or relative standard deviation (RSD; coefficient of variation) of a series of measurements. Precision is the measure of either the degree of reproducibility or repeatability of the analytical procedure under normal operating conditions. The detection limit is a characteristic of limit tests. It is the lowest amount of analyte in a sample that can be detected (i.e., LOD) or quantified (i.e., LOQ) under the stated experimental conditions [14,15].

2.6. Determination of cholesterol content in egg yolk

Cholesterol content in the egg yolk sample was determined by applying 3 μL samples/spot in duplicate along with standards at different concentrations in the quantitative mode of HPTLC analysis. From the calibration curve, the system will generate the concentration of cholesterol/spot. From this value the actual concentration of cholesterol in egg yolk can be calculated by considering the amount used for sample preparation.

3. Results and discussion

3.1. Optimization of the mobile phase and chromatographic conditions

Chromatographic conditions were optimized to develop a sensitive and reproducible assay method for determination of cholesterol content. The mobile phase [i.e., chloroform:methanol (9.5:0.5, v/v)] was found to give a sharp and well-defined peak at Rf of 0.63 ± 0.03. Better resolution was obtained when the chamber was saturated for 20 minutes with the mobile phase at a room temperature of 25 ± 3°C. Thus, this system and aforementioned conditions were selected for the analysis.

3.2. Calibration curve

Specific volumes of stock standard solution were applied for each spot to obtain a range of concentration for each spot (0.1–5 μg of cholesterol/spot). This was carried out in triplicate and repeated for 3 days. For each concentration, the applied spot bands were evenly distributed across the plate to minimize possible variation along the silica layer. The linear regression data for the calibration curve showed good linear relationship over the range of 0.1–3 μg/spot (Fig. 2). Linear regression equation was found to be $Y = 134.659 + 0.894x$ ($r^2 = 0.99972$; standard deviation = 2.19%).

3.3. Validation of method

3.3.1. Precision

The precision of the developed HPTLC method was expressed in terms of %RSD (Table 1). The %RSD levels of intra- and interday precision were found to be less than 1.0 in all cases, which indicated that there were no significant variations in the analysis of cholesterol at these concentrations.
3.3.2. LOD and LOQ
From the regression equation, the LOD and LOQ were found to be 8.09 ng/spot and 24.49 ng/spot, respectively. This value is comparatively better or almost similar to the earlier reported values for other methods of cholesterol detection such as colorimetric, enzyme-mediated biosensor, and polymer microfluidic devices and amperometric systems [16–18].

3.3.3. Selectivity and specificity
The selectivity and specificity of the method were determined by developing an HPTLC chromatogram with solvent blank, complex matrix containing samples, and cholesterol standard. Cholesterol extracted from two different samples, namely, saponified egg and blood serum, was used for the analysis. The solvent blank did not show any spot, whereas the chromatogram of the samples showed clear, compact, and well-separated peaks of cholesterol with an average $R_f$ value of $0.63 \pm 0.03$ (Fig. 3). The method was therefore considered to be specific.

3.3.4. Accuracy and recovery
Accuracy of the method was determined by the standard spiking method by comparing the determined concentration of spiked samples with the theoretical concentrations of the saponified egg yolk sample. Samples were spiked by adding a known amount of cholesterol at different concentrations into the saponified egg yolk samples. For each concentration, spotting was repeated in three different tracks. The mean percentage recovery for each concentration was calculated at each concentration level and reported with its standard deviation. Results showed very good recovery of cholesterol in spiked samples (Table 2).

3.3.5. Determination of cholesterol content in egg yolk sample
Cholesterol fraction extracted from egg yolk, with and without saponification, gave a sharp and well-defined peak of $R_f$ value similar to that of the standard. The concentration of cholesterol was found to be $191.80 \pm 1.83$ mg/egg yolk ($14.95$ g) for the saponified sample saponification, whereas it was found to be $186 \pm 1.29$ mg/egg yolk (approximately $12.44$ mg cholesterol/g yolk) for the unsaponified sample. This value is almost comparable with the measurements obtained using other techniques such as gas chromatography–mass spectrometry (GC–MS) and HPLC analysis. In the study by Malgorzata et al [19], the concentration of cholesterol by GC–MS quantification was found to be $13.91$ mg cholesterol/g yolk of a hen’s egg; Jiang et al [18] reported a value of $11.7$ mg cholesterol/g yolk of a hen’s egg by an HPLC method, whereas Beyer and Jensen [20] reported values of $11.0$ mg/g and $11.7$ mg/g yolk. The concentration of cholesterol in egg yolk may vary depending on

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**Fig. 2** Calibration curve shows linear relation for cholesterol.

| Sample   | Concentration (µg/spot) | Intraday | Interday |
|----------|-------------------------|----------|----------|
|          |                         | Accuracy (%) | Precision (%RSD) | Accuracy (%) | Precision (%RSD) |
| Cholesterol | 2                       | 99.38 ± 0.73  | 0.12          | 99.03 ± 0.52  | 0.23          |
|          | 3                       | 99.27 ± 1.29  | 0.26          | 99.12 ± 0.38  | 0.18          |
|          | 5                       | 98.72 ± 1.09  | 0.39          | 98.81 ± 0.20  | 0.57          |

HPTLC = high-performance thin layer chromatography; %RSD = percentage relative standard deviation.

* Mean of three replicates.
the breed and feed of the chicken. Egg is a highly nutritive food consumed in almost every part of the world. Along with other nutrients, cholesterol is the major component of the egg. Similar to egg, many marketed food materials also contain cholesterol in their composition and their measurements are necessary for determination of balanced diet. Cholesterol content of food materials has recently received far more attention than before due to the increase in cardiovascular disease in humans, especially arteriosclerosis, hypertension, and coronary heart disease.

4. Conclusion

A quick, precise, and accurate method based on normal phase HPTLC has been developed for routine analysis of cholesterol content in different samples. The method was validated for linearity, precision, accuracy, and specificity. It has several advantages over colorimetric and other analytical methods, in that it is cheap, quick, and does not involve complex reactions, and thus is suitable for routine analysis. At any given point of time, multiple samples can be run in a single plate. The average time taken from sample preparation to densitometric evaluation for a single plate is 1.5 hours. Thus, the developed HPTLC method is both less time consuming and cost effective for the determination of cholesterol concentration.

### Table 2 — Recovery of cholesterol showing the accuracy of the method.

| Spiking level | Cholesterol added (ng) | Cholesterol found (ng), mean ± SD, n = 3 | % Recovery (mean ± SD, n = 3) |
|---------------|------------------------|------------------------------------------|-------------------------------|
| 0             |                        | 1908 ± 1.862                             |                               |
| 1             | 300                    | 2187 ± 2.130                             | 99.04 ± 0.41                 |
| 2             | 500                    | 2421 ± 1.074                             | 100.53 ± 0.28                |

SD = standard deviation.

### Conflicts of interest

The authors declare that there are no conflicts of interest.

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