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# Cholesterol Removal from Milk Nvith Crossed linked B-cyclodextrin and Determination of Cholesterol by High Performance Liquid Chromatography

**Sumeet Dhondge[1!] and Borkar Motiram[2!]**

[1]Research Scholar, Department of Zoology, Digambarrao Bindu Arts Commerce and Science College Bhokar, Dist. Nanded, India
[2]S.R.T.M. University, Nanded
Email: dhondgesumeet123@gmail.com[1] | motiramborkar123@gmail.com [2]
!Authors equally contributed in wet lab and manuscript preparation

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## Abstract

A sensitive and high-performance liquid chromatographic method has been developed to determine the cholesterol content in milk and dairy products. A comparison was made between acetonitrile: 2-propanol (8:1, v/v), acetonitrile: methanol (3:1, v/v), and acetonitrile: methanol: 2-propanol (7:3:1, v/v) for optimizing cholesterol separation. In comparison with other mobile phase systems for separating cholesterol, acetonitrile/methano1/2-propanol was the best. To remove interfering compounds, the liquid-liquid extraction (LLE) of cholesterol was simplified with hexane, a nonpolar solvent that has a high recovery (100±1.0%) of cholesterol. Sep-pak Cl8 was used to develop a solid phase extraction (SPE) method that was compared with liquid phase extraction (LLE).There was high reproducibility and rapidity in the SPE method. In combination with saponification of esterified cholesterol, both extraction methods were useful to determine total cholesterol. It was possible to detect cholesterol at a level of 0.01 iμg. This new HPLC method has advantages over traditional methods because it is simple, rapid, and accurate.

**Keywords:** HPLC , cyclodextrin, saponification

## 1. Introduction

In recent years, cholesterol, the main component of unsaponified milk fat, has been gaining popularity. Due to its possible relationship to diseases such as anthersclerosis, it drew considerable attention during the year. Milk and milk products have been associated with several studies regarding cholesterol content [1]. Estimation of cholesterol has been done using gravimetric measurements using a colorimeter based on saponification of milk fat. To estimate cholesterol from milk, Reinhold and Shield zero modifications were applied. In this paper, we present our findings on the application of this method for determining cholesterol in milk. It has the chemical structure of chole-bile and stereos. solid (Greek chole-bile and stereos solid).

An essential component of the membrane of all animal cells, it is biosynthesized by animal cells as sterols (or modified steroids). Myant [2]. Animals synthesize cholesterol primarily to synthesize steroid hormones, bile acid, and vitamin D, as well as in the structure of animal cells.

In Most of the hepatic cells are produced by vertebrates. Myropicisma, which requires cholesterol for growth, is an exception to the cholesterol absence among prokaryotes.

Gallstones were first identified as solid forms of cholesterol by Francois Poulletier de la Salle in 1769 [3, 4]. A chemist named the compound cholesterine in 1815, but it wasn't until then that Michel Eugene Chevreul gave it its name. Lowe [5] Increasing awareness and publicity about the relationship between plasma cholesterol and coronary heart disease has led to an increase in public interest in cholesterol. A number of factors contribute to the development of heart disease, but hypercholesterolaemia, or high blood cholesterol, is one of the most important. Lipoproteins are particles that carry cholesterol through the bloodstream. Having too much cholesterol in the blood can damage arteries, especially coronary arteries [6]. If a blood-clot completely obstructs blood flow to the heart, chest pain (angina) results. The cholesterol-laden 'plaque" builds up in vessel walls, causing a condition called atherosclerosis [7].

Heart attacks (myocardial infarctions) or death may result in coronary arteries already affected by atherosclerosis [8]. Therefore, factors known to raise plasma cholesterol, like food cholesterol, are generally regarded as unfavorable. The importance of determining cholesterol levels in food materials is underscored by this situation. It is found in milk fat to contain about 025-0.40% cholesterol. Cholesterol is present in foods derived from animals, but not in foods derived from plants.

In industrialized countries, coronary heart disease accounts for around 25% of all deaths. First recognized as a clinical condition a century ago, heart attacks are a relatively new phenomenon. Adolf Windaus, a German chemist, discovered in 1910 that cholesterol tends to mass in atherosclerotic plaques [9]. As a result of hereditary hypercholesterolemia (FH), a Norwegian physician described it in 1938, resulting in significantly higher blood cholesterol levels in middle age, and a massive increase in heart attack risk. LDL and HDL were separated from blood plasma by John Gorman, a doctor at the University of California, in 1955. In addition, he found significantly lower levels of HDL in plasma from patients with heart attacks than those without [9]. Murakami et al. [10] predicted that people who move from low fat consumption communities to high fat consumption communities will have the same plasma cholesterol levels as those who move from low fat consumption communities to high fat consumption communities. Several acetate molecules are polymerized repeatedly to produce cholesterol indirectly, and Konrad Bloch and Feodor Lynen won Nobel Prizes in 1964 for their discovery of 3-hydroxy-3 methyl glutarate attached to CoA (HMG CoA), which is involved in cholesterol synthesis. As a result of this discovery, researchers focused on regulating HMG CoA reduciase, an enzyme that controls cholesterol synthesis rate [10].

In the past, extensive research has been conducted on determining the cholesterol content of foods. Cholesterol in blood plasma can be estimated using several methods, some of which are modified for dairy products and foods [11]. For ghee and fat-rich dairy products, however, there are only limited methods available for estimating cholesterol content. These methods either require hazardous chemicals or expensive equipment and are cumbersome or require cumbersome procedures. Also, it is important to keep in mind that specific chemicals, such as acetic anhydride, are not readily accessible due to the Narcotic Drugs and Psychotropic Substances Act (NDPS Act, 1985), which prohibits specific chemicals from being used in Liehermann- Burchard reagents for cholesterol estimation. The removal of acetic anhydride from the process necessitated the development of an alternative method that was not only non-hazardous but also easy to use.

**Cyelodextrins**

An unidentified crystallized substance was discovered in 1891 when Villiers reported the fermentation of starch. A French author named this substance "cellulosine" based on his assumption that it was some type of cellulose [12]. After 15 years, Schardinger found that when cultivated on starch medium, Bacillus macercins produces two distinct crystalline substances: one crystalline substance and one crystalline substance that reproduces. Due to their similar properties to partial degradation products of starch, “Freudenberg and his colleagues elucidated the cyclic structure of these two dextrins in the mid 1930s”. CDs were discovered between 1891 and 1936, which is known as the discovery period.

**Structures of cyclodextrin**

In the family of cyclic oligosaccharides, also known as cyclodextrins, there are several rare and minor cyclic oligosaccharides. In three major CDs, gluocopyranoses are crystalline, homogeneous, and non-hygroscopic. Six gluoco-

pyranose units are found in -cyclodextrins (Schardinger's -dextrin, cyclomaltohexose, cyclohexaglucan, cyclohexa-amylose, CD, BCD, C7B), -cyclo-dextrins (Schardinger's -dextrin, cyclornalio-heptaose, cycloheptaglucan, cycloheptaa-mylose, -CD, BCD, C7B) consisting of seven gluocopyranose units (CD, BCD, C7B) and -cyclodexirins (Schardinger's -dextrin, cycloctaglucan, cycloheptaamylose) consist of eight gluocopyrins, C8A) consist of eight gluocopyranose.

High performance liquid chromatography is a technique used to determine cholesterol levels in foods using saponified or esterified chemicals [14]. Various mobile phases can be used in reverse phase systems, including propanol/hexane and methanol. Short-wavelength UV light is commonly used for HPLC to detect cholesterol (200-210 nanometers. A number of liquid chromatographic methods using UV-absorbing derivatives have been developed because of their simplicity and wide range of applications.

Holen (1985) investigated reversed phase HPLC as a method for separating free sterols. 0.8 M ethanolic KOH was used to saponify rape seed oil and mayonnaise for 30 minutes at 80°C. After extracting the unsaponitiable fractions six times in ether, they were treated with anhydrous Na 2 sSO4 and evaporated to dryness. 0.1 μm Millipore filters were used to filter the residues dissolved in absolute ethanol. Using two different mobile phases, methanol and water (99:1) and methanol and water (85:15), filtrates were injected into two reversed phase columns. A UV detector at 206 nm was used to elute samples based on decreasing polarity. Cholesterol can be detected as low as 0.4 μm using this method. For quantifying cholesterol from egg yolk lipids, Helen (1987) used an HPLC system.

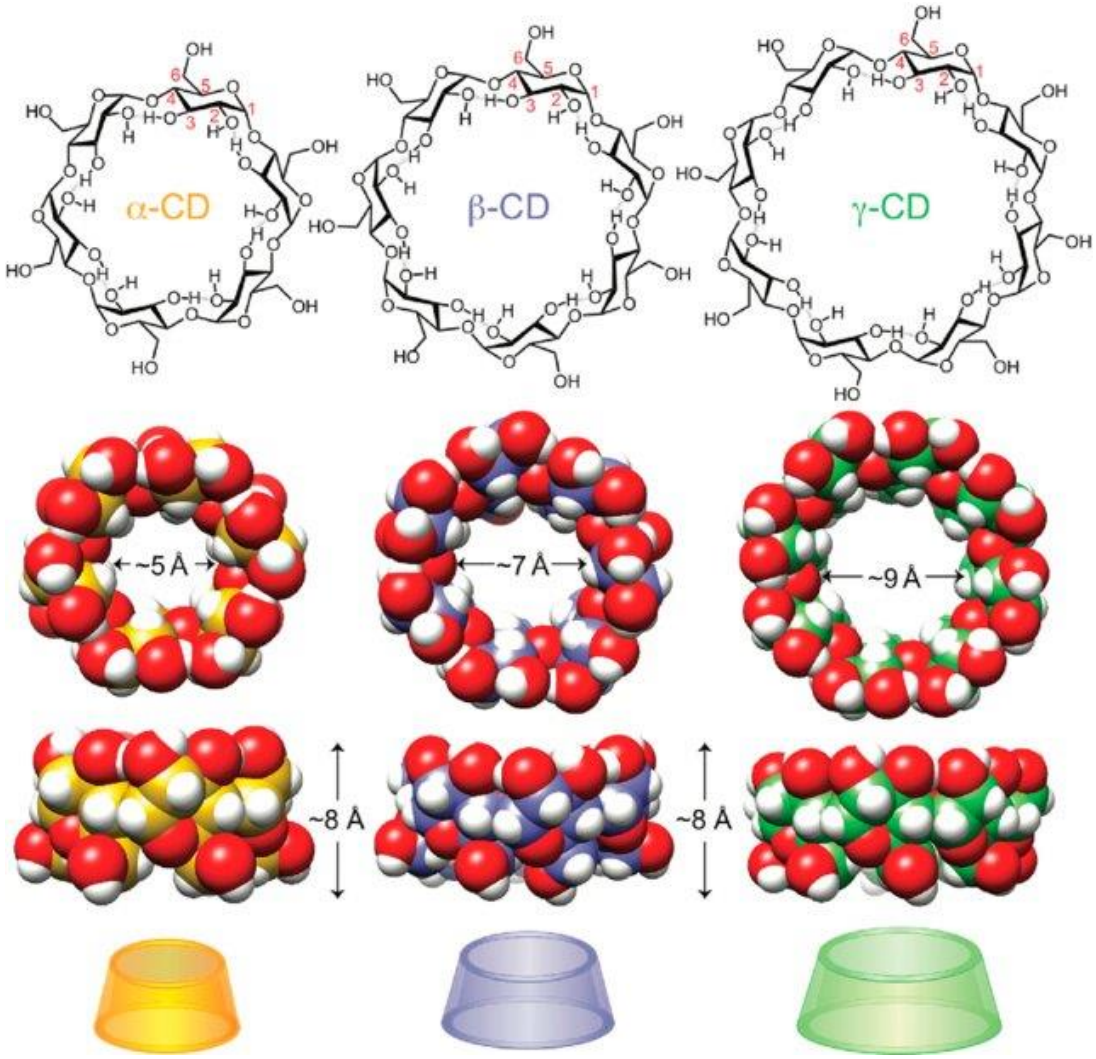


**Figure 1.** Structural, space-filling, and graphical representations of the three main cyclodextrin (CD) molecules: α-CD (yellow), β-CD (blue), and γ-CD (green).

*Source* :Carson J. Bruns, Symmetry 2019, 11, 1249; doi:10.3390/sym11101249 [13]

HPLC loops were loaded with cholesterol in 0.1 μm to 5 μm concentrations along with cholesterol extracted in hexane and injected into loops with 2 to 250 mg levels. Cholesterol was detected at 210 nm by using a UV detector after it had been quantified with 0.75% IPA in hexane. Egg yolk lipids spray dried using this method contained 3048 mg of cholesterol per 100 grams.

Liquid chromatography was used to determine milk and milk products' cholesterol content. The aim of this study was to develop a method for separating cholesterol by HPLC by comparing different extraction processes and mobile phases. Oh*et al* (2001). Solid phase extraction (SPE) yields higher recovery rates and shorter extraction times than liquid-liquid extraction (LLE). Furthermore, acetonitrile: methanol: 2-propanol separated cholesterol better than the other mobile phase systems. HPLC was conducted using a C18 column and a 20 l injection loop using a multi solvent delivery pump. In 205 runs, elution of cholesterol was monitored using a UV detector at 1.6 mlimin of mobile phase flow

**Cholesterol's chemical properties and health-related functions:**

All animal tissues and blood plasma contain cholesterol, a waxy substance. As an organic compound, cholesterol belongs to the family of steroid compounds. its molecular formula is $C_{27}H_{46}O$. An odorless, tasteless, white, crystalline substance, it is odorless and tasteless when pure. Our bodies require cholesterol to function properly. It is also the basis of bile acids, steroid hormones, and vitamin D, and is found in the membranes of all cells. The liver and other organs synthesize cholesterol in the bloodstream. Humans also consume a lot of cholesterol. As a result of a compensatory system, the liver produces less cholesterol when dietary cholesterol levels increase.

High cholesterol levels in the bloodstream are a major cause of atherosclerosis. Fat deposits can build up inside blood vessels when there is insufficient cholesterol in the blood. These deposits accumulate fat as well as thicken. As a result, the vessel walls become calcified, eventually resulting in scarring. Heart attacks and strokes are caused by the narrowing of blood vessel channels caused by deposits. People with high cholesterol levels "more than 240 mg of cholesterol per 100 cc of blood plasma" are more likely to develop coronary heart disease as cholesterol deposits accumulate in their vessels.

A lipoprotein is a protein complex that binds cholesterol and transports it through the bloodstream. As cholesterol is transported from the liver to the tissues and cells in the body by low-density lipoproteins (LDLs), it is separated from lipoproteins and can be utilized. As bile acids are excreted from the liver, excess cholesterol can be transferred from the tissues to the liver via high-density lipoproteins (HDL). LDLs are primarily responsible for forming atherosclerotic deposits in blood vessels. Atherosclerosis, however, may actually be retarded or reduced by it.

## 2. Review of Literature

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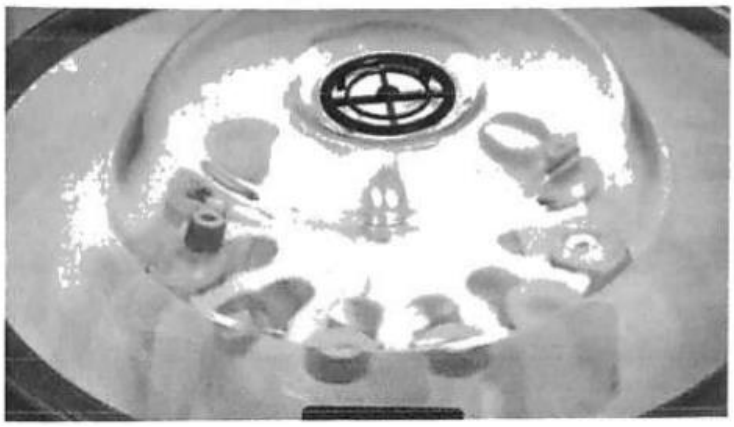


**Cholesterol: high-density lipoproteins and low-density lipoproteins**

**Cholesterol structure and properties**

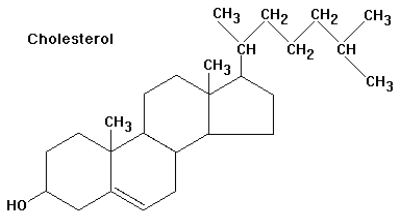
A cholesterol molecule consists of two parts, which are called chole and stear, respectively, which mean "bile" and "hard fat." Michel Chevreul rediscovered cholesterol in 1818 and named it cholesterine, believing it to be a fat. In its pure form, cholesterol looks like flakes and is chemically a fat-like substance. A cyclo-hexane ring is made up of six carbon atoms; it contains a cyclo-pentane ring made up of five carbon atoms, as well as a side chain of eight carbon atoms. It has a molecular weight of 386.66 and it has the chemical formula $C_{27}H_{46}O$. An esterification of cholesterol's polar hydroxy l group to a fatty acid produces cholesterol ester, which has a slightly hydrophilic nature. The hydrophobic and hydrophilic regions of cholesterol also influence membrane fluidity. There is no soluble form of cholesterol in water. A cold alcohol or petroleum ether will only sparingly dissolve it, and a hot alcohol will completely dissolve it.

Sublimates and distills under high vacuum at 48.50C. Among all animal cells, cholesterol is especially abundant in nervous tissue. Cholesterol is used by the body for a variety of purposes, including making cell walls and hormones.

**Function of Cholesterol**

When cholesterol is present at its ideal level, it has many beneficial functions; however, when it is present at high levels, it may pose a danger to the heart. In the human body, cholesterol is required for various tissues, bile acids, and hormones. Several cell membranes, including skin, intestines,

nerves, and brain, contain cholesterol, but its exact role is unknown. As much as 80% of the cholesterol in human bile is synthesized by the liver into cholic acid. Glycine and taurine combine with bile acids to form water-soluble salts that are powerful emulsifiers during fat digestion. With its carbon atoms containing 21, 19, and 18 respectively, cholesterol is the chemical ancestor of hormones such as progesterone, cortisone, and estrogen. The ovaries, testes, and adrenal glands produce these hormones. Vitamin D is also synthesized from cholesterol.




## 3. Methodology

**Removal of Cholesterol from Milk Sample withcrossed Linked Beta Cyclodextrin by Adipic Acid**

**Preparation of Beta Cyclodextrin**

A 100 g sample of $\beta$-CD was dissolved in 80 ml distilled water and incubated at room temperature for 2 hours with constant agitation. After incorporating 5% adipic acid with 100 $\beta$--CD, 1 N NaOH was used to adjust pH to pH 10 for 90 minutes before acetic acid was used to adjust pH to 5. $\beta$ Filtered Whatman paper No. 2 was used to recover CDs, and 150 rriL of distilled water was used to wash them three times. A labline mechanical convection oven was used to dry the product for 20 hours at 60°C.

**Cholesterol removal**

To In order to investigate the effects of different factors, the contents of a 1,000 mL beaker were weighed and $\beta$-CD was added in concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 %. With different mixing temperatures, mixing speeds, and mixing times (0, 5, 10, 15 or 20°C), the mixture was stirred at 800 rpm with a blender (Tops; Misung Co., Seoul, Korea). A centrifuge (HMR -2201V; Hanil Industrial Co., Seoul, Korea) was used to centrifuge the mixture for 10 minutes. To determine cholesterol levels, the supernatant fraction of treated milk was decanted after centrifugation and analyzed.

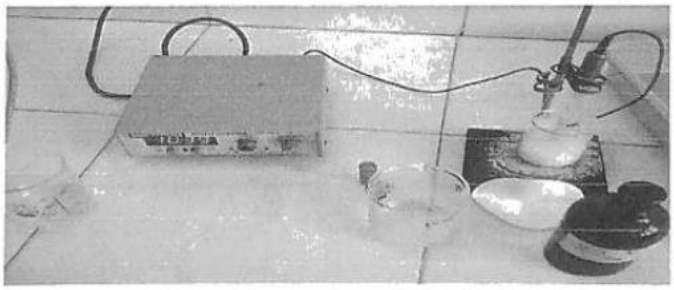


**Figure. pH meter**

**Purification of supernatant and determination of cholesterol by TLC and column chromatography**

**Column chromatography**

In Prior to further fractionation, our procedure can be applied to most lipid mixtures, for example by HPLC or another low pressure chromatography technique, or by thin layer chromatography.

**Apparatus:** Solvent flow is controlled by a sop cock at the bottom of a glass column with an inside diameter of 8-10 mm and a length of 20 cm. Chloroform, methanol, and acetone were used as reagents for silica gel 60 (230-400).

**Procedure:**

1. Using 2 or 3 ml chloroform, prepare a slurry of 300mg silica gel.
2. Chloroform should be injected into the column after the solvent is slowly drained through the stopcock.
3. Drop the solvent level of the gel to the top
4. Chloroform the supernatant sample and place it on top of the gel
5. Pure samples containing chloroform will be eluted by this method

**Figure:** Column chromatography for cholesterol purification from supernatant

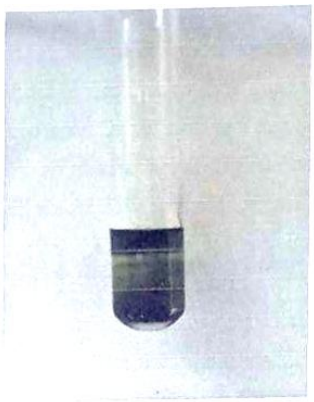


**Figure:** Analysis of presence of cholesterol in purified sample

**2. TLC (thin layer chromatography)**

Silica gel was used as the stationary phase (adsorbent) and the following solvents were used: petroleum ether, normal hexane, chloroform, ethyl acetate, and glacial acetic acid.

**Method for the Determination of Cholesterol in Milk Sample by Liebermann-Burchard Reaction**

**Reagents**

(1) Ethanol-ethermixture: 3 vol. 95% ethanol are mixed with 1 volether.
(2) Anhydrous chloroform.
(3) Acetic anhydride (Analar).
(4) Sulphuric acid (Analar).
(5) Cholesterol standard

**(a) Stock solution: dissolve 0-547 g. anhydrous cholesterol in 100 ml. chloroform**

A volumetric flask was filled with chloroform to a volume of 50 ml after 250 mg of cholesterol was dissolved in chloroform.

**(b) Single working standard: prepare a 1 in 50 dilution of (a) in chloroform. (10 ml. = 250 mg. total cholesterol/100 ml.)**

An acetic acid working solution containing 100 grams of cholesterol per ml was prepared from a ml of stock solution diluted to a volumetric flask in 50 grams of acetic acid. Adding sulphuric acid slowly (1 ml) to Lambert Burchard reagent. Mix chilled acetic anhydrous (20) with oc for 27 minutes.

**Unknown prepared: -**

1 ml Purified sample by column chromatography dissolved in 3 ml of chloroform. This was then mixed properly with 4 ml of Liebermann- Burchard reagent.

A water bath was used to incubate the mixture at 250C for 12 minutes. It took three minutes to determine the optical density of a blank reagent after incubation.

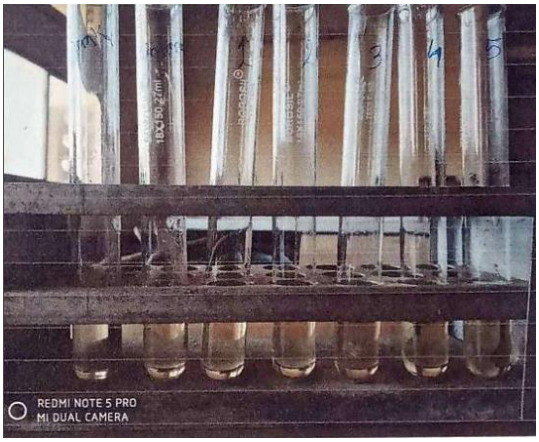



**Fig.** analysis of unknow sample

**Observation table:**

| Test tube | Amount of sample (ml) | Amount of chloroform | Concentration of cholesterol(ml) | Reagent (ml) | | O.D at 640nm |
|---|---|---|---|---|---|---|
| 1 | Blank | 0 | 0 | 4 | | 0.00 |
| 2 | 0.2 | 0.8 | 100 | 4 | | 0.168 |
| 3 | 0.4 | 0.6 | 200 | 4 | **Incubation for 30 minutes** | 0.191 |
| 4 | 0.6 | 0.4 | 300 | 4 | | 0.193 |
| 5 | 0.8 | 0.2 | 400 | 4 | | 0.229 |
| 6 | 1.0 | - | 500 | 4 | | 0.254 |
| 7 | Unknown | 0.9 | - | 4 | | 0.180 |

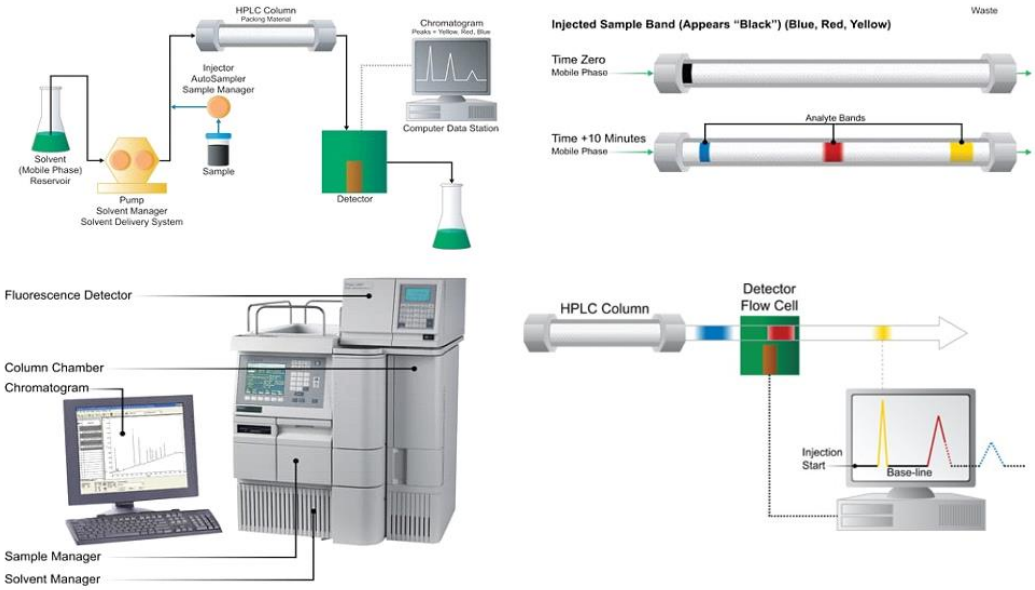



**Figure:** High performance liquid chromatography

Column: C18 ODS, Mobile phase: 1ml/min, Flow rate: 1 ml/min, Detection wavelength: 215nm
Sample prepared in Chloroform and Methanol

**Preparation of standard solution:**
A volume of 10ml was prepared by dissolving 10mg of cholesterol in 5ml chloroform. From the above solution, 1 ml was accurately measured and diluted to 10 ml chloroform. A concentration of 1 µg/ml was obtained by dilution with methanol of the accurately measured 0.1 from the second solution.

**Preparation of sample solution:**
Chloroform (S1) was used to dilute 0.1 ml of the original sample solution to 10 ml, followed by methanol (S2) to further dilute 0.1 ml further to 10 ml. Methanol (S2) was used to further dilute 0.1ml of the above solution to 10ml

**Calculation:**
Concentration Cholesterol in given sample WEIS calculated as Follows

$$Ct = \frac{At}{As} x Cs$$

$$Ct = \frac{66653425 \times 1}{66337509}$$

Where,
Ct =. Concentration of test solution
Cs = concentration of standard solution (S1)
At = peak area of test (S2)
As = Peak area of standard
Conc. Of original sample = Ct x D1 x D2
D1 = Dilution factor of first diluted sample solution
D2 = Dilution of second diluted sample solution

**Observation table:** Peak area standard and test solution of cholesterol

| Sr. No. | Conc. Of cholesterol solution | Peak Area |
|---|---|---|
| Std. cholesterol | 1 µg/ml | 66356947 |
| | 1 µg/ml | 66454012 |
| | 1 µg/ml | 66201569 |
| | 1 µg/ml | 66337509 |
| | Average | 66337509 |
| Sample | Replicate 1 | 66745250 |
| | Replicate 2 | 66561601 |
| | Average | 66653425 |

## 4. Discussion

In order to minimize the need for expensive, hazardous, and environmentally objectionable solvents and reagents, a simple, rapid, and precise method for determining cholesterol in milk and dairy products has been developed and evaluated. Liquid-liquid extraction (LLE) and solid phase extraction (SPE) are two extraction methods that were evaluated. Biological molecules form complexes with cholesterol. These changes change the physical properties of phospholipids and proteins, which makes cholesterol extraction inefficient.

It As a result of the work I did based on this method, cholesterol determination from milk powder can be easily adopted. In the presence of crossed linked B-CD prepared with adipic acid, the cholesterol removal rate varied from 92% to 93% depending on the mixing conditions (temperature, time, and speed). It can be useful to know the cholesterol content of milk and food products from such a study. HPLC, a newly developed method, has the advantages of being fast, simple, and accurate over many other methods.

**Conflicts of interest:** The author stated that no conflicts of interest.

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