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THE COMPARISON OF HPLC AND SPECTROPHOTOMETRIC METHOD FOR CHOLESTEROL DETERMINATION

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ABSTRACT

The present study was carried out to compare two different analytical methods (HPLC and spectrophotometric) for determination of cholesterol content in milk while cholesterol in food is important not only for the nutritional value setting of foods but also due to the validation of a fast, reliable and economical method for studying the possible mechanism of its reduction. Spectrophotometric determination of cholesterol content was based on the Liebermann-Burchard (LB) reaction among cholesterol, ethyl acetate, acetic anhydride, plus concentrated H_2SO_4 and measuring absorbance of formed color at 625 nm. HPLC method was performed by column chromatography on reverse phase C_{18} with DAD detection at 205 nm. The methods were applied to the milk sample. The achieved LOD and LOQ for HPLC were 2.13 mg.kg^{-1} and 6.45 mg.kg^{-1}, respectively, while for spectrophotometric method were 12.55 and 38.04 mg.kg^{-1}. The difference between cholesterol content determined by both methods was statistically insignificant at $p < 0.05$. Therefore, it can be concluded that both methods are suitable for determination of cholesterol content in milk, however, HPLC method exhibited higher sensitivity and lower limits of detection or quantification, respectively.

Keywords: cholesterol; HPLC; spectrophotometry; analysis; milk

INTRODUCTION

Cholesterol is a key compound in most biological systems. It is an essential compound in cellular membrane functions of animals and the precursor of important endogenous substances. In humans, cholesterol is obtained from two sources: endogenous synthesis and exogenous ingestion from food (**Ramalho, Casal and Oliveira, 2011**).

From a nutritional point of view, cholesterol is not found in significant amounts in plant sources, is mostly present in foods of animal origin, namely cheese, egg, beef, pork, poultry, fish, and shrimp. High levels of low-density lipoprotein cholesterol are a major cardiovascular risk factor. Once dietary cholesterol intake is increasing, the plasma cholesterol levels rise and consequently increases the risk of cardiovascular diseases and atherosclerosis (**Albuquerque et al., 2016**).

Multiple methods have been developed for cholesterol levels determination. According to **Li et al. (2019)** the methods can be divided into three major categories: 1. classical chemical methods based on the Abell-Kendall protocol, 2. fluorometric and colorimetric enzymatic assays, and 3. analytical instrumental approaches. Cholesterol determination procedures in foods usually involve lipid extraction, separation of cholesterol from interfering components or liberation of cholesterol into the free form, and measurement of isolated cholesterol. A mixture of polar and nonpolar solvents has been suggested to give better cholesterol extraction from food materials because cholesterol in these samples is usually bound by many other biological compounds such as lipoproteins, proteins, and phospholipids, and a multiple extraction approach was thought to be more suitable to remove membrane cholesterol (**Dinh et al., 2011**). Gas and liquid chromatography are the most suitable methods for cholesterol determination, due to their ability to separate and quantify this compound from other similar ones (**Albuquerque et al., 2016**). The foremost colorimetric test for the identification of cholesterol is probably the Liebermann-Burchard (LB) reaction, which was first described in 1885 (**Xiong, Wilson and Pang, 2007**). It includes saponification of cholesterol ester with alcoholic potassium hydroxide, extraction of hydrolyzed cholesterol with hexane followed by evaporation of the solvent, and finally color development with acetic anhydride and concentrated sulfuric acid. However, its use is not accepted for routine tests nowadays since highly corrosive reagents are used (**Li et al., 2019**). High-performance liquid chromatography (HPLC) has the main advantage of being carried out at relatively low temperatures, thus preventing cholesterol oxidation (**Ramalho, Casal and Oliveira, 2011; Albuquerque et al., 2016**). In spite of some

drawbacks, such as elevated volumes of solvents and limits of detection and quantification, sample preparation is simple and required a small number of steps (saponification and the choice of extraction solvents are needed for adequate separation and quantification of analytes by HPLC) (**Bauer et al., 2014**).

Scientific hypothesis

Both HPLC and spectrophotometric method could be acceptable for the determination of cholesterol content in milk.

MATERIAL AND METHODOLOGY

All reagents and standards were of analytical grade. Cholesterol standard was from Sigma-Aldrich with a purity ≥99%. Potassium hydroxide (KOH), concentrated sulfuric acid (H_2SO_4), and acetic anhydride were purchased from Mikrochem (Pezinok, Slovakia). Ethyl acetate, n-hexane, and sodium sulphate anhydrous were purchased from Centralchem s.r.o. (Bratislava, Slovakia). Methanol, HPLC grade was purchased from Fisher Chemical (Loughborough, UK). The cow´s milk (3.5% fat, Tatranská mliekareň a.s., Kežmarok, Slovakia) was bought in a local market.

Sample preparation
HPLC analysis

The samples were prepared according to the modified method of **Borkovcová et al. (2009)**. To the 5.0 g of the sample methanolic solution of KOH (1 $mol.L^{-1}$) was added and refluxed for 30 min. After cooling, 10 mL of n-hexane and 5 mL of deionized water were added and intensively shaken in a separating funnel. The organic layer was separated into the beaker with 2.0 g of sodium sulphate. The water layer was further washing 2 more times. The hexane solution was evaporated, and the residue was dissolved in 3 mL of ethyl acetate. The solution was filtered using syringe filters with PVDF membrane and particle size 0.45 µm (Agilent Captiva, USA). The prepared solution was directly analyzed by HPLC chromatograph. The calibration curve was performed using seven standard concentrations. A stock solution of cholesterol (1 $mg.mL^{-1}$) was diluted in methanol to prepare calibration standards at 25, 40, 50, 75, 100, 300, and 350 $\mu g.mL^{-1}$.

Spectrophotometric determination

The samples for spectrophotometric determination of cholesterol were prepared similarly to HPLC analysis. The LB color reagent was prepared according to the modified method of **Xiong et al. (2007)**. Ethyl acetate (75 mL), acetic anhydride (60 mL), and concentrated H_2SO_4 (12 mL) were pipetted to the volumetric flask at 0 °C, stirring for 10 min, and storage in the fridge for 3 hours. To the prepared LB reagent 1 mL sample solution was added. After 5 min the absorbance value was recorded at 625 nm for 20 min. The concentration of cholesterol in the sample was calculated from the calibration curve, which was performed using calibration standards. The calibration standards were prepared by dilution of cholesterol in ethyl acetate at 0.1 to 1 mg.

Instrument and chromatographic conditions
HPLC

Chromatography analysis was performed using an Agilent Technologies 1260 infinity system (USA) equipped with a vacuum degasser, a quarterly pump, an autosampler, and the UV-DAD detector. Cholesterol was detected at UV wavelength of 205 nm. Isocratic elution was performed at a flow rate of 1.2 $mL.min^{-1}$ using the mobile phase consisted of water/methanol 5:95 (v/v). The injection volume was 10 µL and the temperature was set at 35 °C. As a stationary phase, a Poroshell 120 EC-C18 column (4.6 x 50 mm, 2.7 µm particle size) was used with the guard column Poroshell 120 EC-C18 (4.6 x 5 mm, 2.7 µm particle size). The results were recorded using the OpenLab CDS software, ChemStation Edition for LC and LC/MS systems (product version A.01.08.108).

Spectrophotometric determination

Spectrophotometric determination was performed using a spectrophotometer Cary 300 UV-Vis (Agilent Technologies, USA). The detection wavelength was 625 nm. The results were determined with Cary WinUV software (software version 4.20(468).

Statistical analysis

Results are expressed as mean ±standard deviation or as percentage. Statistical analysis was performed using Microsoft Exel version 2010. The data were subjected to the Student´s test and the values were considered significantly different when $p < 0.05$.

To obtain validation parameters, the linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, and selectivity were determined. The linearity was evaluated according to the correlation coefficient by Pearson (R^2) for linear regression. The LOD and LOQ were calculated considering the signal-to-noise ratio accepted for each limit and the parameters estimated for the analytical curve, according to equations 1 and 2:

$$LOD = 3.3 \times \frac{s}{S} \qquad (1)$$

$$LOQ = 10 \times \frac{s}{S} \qquad (2)$$

Where s is the estimate of the standard deviation of the equation's linear coefficient, and S is the angular coefficient of the analytical curve (**Bauer et al., 2014**).

The precision was assessed by the Horrat test, which is the ratio of the method standard deviation and the Horwitz relative standard deviation (equation 3):

$$RSD_{Horwitz} = 2^{(1-0.5logC)} \qquad (3)$$

Where C is the analyte concentration in mass percentage (**Ribeiro and Brandão, 2017**).

The accuracy was evaluated by recovery studies at one standard concentration level of cholesterol (1 $mg.mL^{-1}$). Recoveries were evaluated by adding to milk sample aliquots standard solutions of the analytes. After the quantification of the analytes in the fortified samples and in the control, the recovery percentage (% REC) was calculated according to equation 4 (**Bauer et al., 2014**):

$$\%REC = \left(\frac{Obtained\ conc. - Control\ conc.}{Expected\ conc.} \right) \times 100 \qquad (4)$$

Selectivity was evaluated by using the spectra provided by the DAD detector by comparison of the peaks present in the chromatograms of the products with those peaks in the chromatograms of the standards, as described by **Bauer et al. (2014)**.

In order to evaluate the conformity of the results obtained by HPLC and spectrophotometric determination, Moore´s test was used according to **Eckschlager, Horsák and Kodejš (1980)**. The test is applicable if $n_A \neq n_B$ and the range of R_A and R_B is used as a measure of variance. Conformity is tested according to Moore´s criterion (U). Moore´s criterion is calculated according to the equation 5:

$$U = \frac{|\overline{x_A} - \bar{x}_B|}{R_A + R_B} \tag{5}$$

Where x_A is an average value obtained from the first method, x_B is the average value obtained from the second method, and R_A, R_B are the values of variance. The calculated U is compared with the critical value U_α. If $U \geq U_\alpha$, the difference is statistically significant at $p < 0.05$. If $U < U_\alpha$, the difference is not significant and we accept the null hypothesis about the consistency of the results (**Eckschlager, Horsák and Kodejš, 1980**).

RESULTS AND DISCUSSION

Optimization of the spectrophotometric determination and chromatographic conditions

In color-based methods, the application of the LB reaction is usually the key step after the extraction procedure (**Dinh et al., 2011**). Cholesterol in the presence of concentrated sulphuric acid and acetic anhydride is oxidized to a conjugated pentaene known as cholestapolyene carbonium ion and this undergoes further reaction to form cholestahexaene sulphonic acid, with a wavelength of absorption of 410 nm (**Adu et al., 2019**). The LB reaction depends, however, on various factors, such as temperature, time, proportions of reactant, wavelength or exposure of light as described by **Kenny (1952)** or **Essaka (2007)**. Firstly, our study thus investigated the kinetics of LB reaction. We monitored the dependence between the time of reaction and the absorbance of the solution. The results are shown in Figure 1. The absorbance maximum at 625 nm is stable for

20 to 30 min and there is little difference in the measured absorbances. With the increasing time, the absorbance maximum is moving to higher wavelength values (665 to 670 nm), where is also stable. However, a longer time interval is less suitable regarding total analysis time. From the Student´s test, it was observed that the difference between the absorbance values at 625 nm in 20 and 30 min was not significant at $p < 0.05$. The spectrophotometric measurement was thus optimized regarding these results. **Atinafu and Bedemo (2011)** used quite a similar wavelength (640 nm) for the determination of cholesterol in some commercial edible oils. According to **Burke et al. (1974)**, a 30 min reaction time is optimum for spectrophotometric measurement. According to **Kim and Goldberg (1969)**, maximum color development occurs after 15 – 18 min incubation at 30 °C. The other important factor, which has to be considered, is the stability of LB color reagent. **Kim and Goldberg (1969)** stated that the LB reagent is not unstable and it need not be used within a few hours. According to these authors, the reagent is stable for 6 months when stored at 4 °C. On the other hand, some authors using the LB reagent, which was prepared freshly (**Sperry and Brand, 1943; Xiong et al., 2007; Adu et al., 2019**). Firstly, the stability of LB reagent was measured after 7 hours. After this time the new calibration standards curve was recorded. Based on the Student´s test the differences were statistically insignificant at $p < 0.05$. Statistically insignificant differences were also noticed after 24 and 48 hours. From the results, it was thus obvious that LB color reagent is stable. The reaction is also influenced by the stability of cholesterol solution. The difference between the results obtained with the freshly prepared cholesterol solution and after 21 days was statistically significant at $p < 0.05$ thus the solution was not stable, and the use of freshly prepared solution is recommended.

Because of the slight polarity caused by the hydroxyl group, either normal-phase (NP) or reversed-phase (RP) HPLC can be used for the analysis of cholesterol (**Dinh et al., 2011**). In our study, we worked with non-polar C_{18} stationary phase and polar mobile phase.
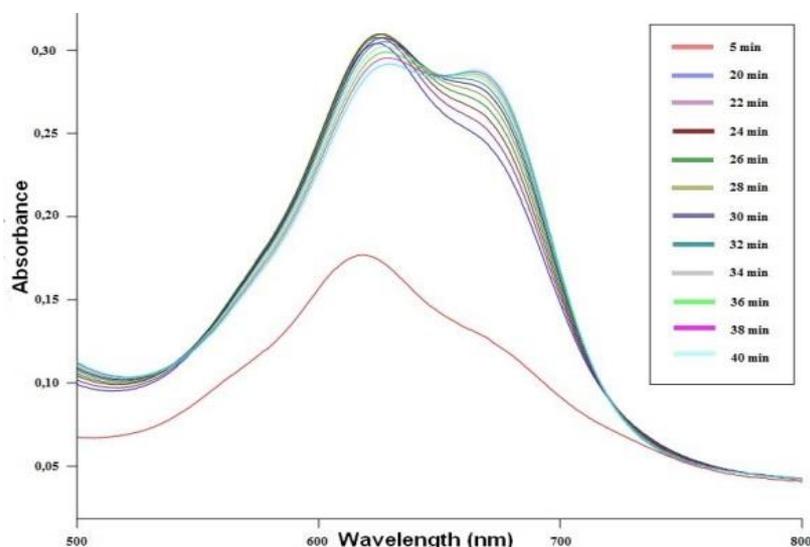

Figure 1 Absorbance spectrum of Liebermann-Burchard reaction.

In literature, there is described a lot of different types of mobile phase composition. For example, **Borkovcová et al. (2009)** used water with methanol 5:95, **Oh, Shin and Chang (2001)** acetonitrile: methanol: isopropanol 7:3:1 or **Bauer et al. (2014)** acetonitrile with isopropanol 95:5. In this work, several mobile phases were tested but the best results were obtained using deionized water with methanol (5:95, v/v). The same conditions were described by **Borkovcová et al. (2009)**. After the optimization procedure, the retention time of cholesterol peak was 5.2 min. The absorbance spectrum (Figure 2) showed that the maximum is obtained at 201 nm, but to avoid the interferences caused by impurities, we used the absorption at 205 nm, because the differences were not statistically significant $p < 0.05$.

Sample analysis and validation

The sample saponification and extraction of cholesterol to non-polar solvent were crucial steps for the analysis of this compound in milk by both methods. The saponification of the lipids has the primordial objectives of removing acylglycerols from the extract of the lipids and hydrolyzing the esters of cholesterol. The reaction can be done after the extraction of the lipids, or by direct saponification **(Bauer et al. 2014)**. These authors also suggested that direct saponification is preferably due to a significantly lower quantity of solvents and shorter preparation time. In our work, we thus used direct saponification followed by the extraction. According to **Ahn et al. (2012)**, three important factors must be considered when selecting a cholesterol extraction solvent: a high solubility of cholesterol, a low efficiency for fat extraction, and hydrophilicity. The most widely used solvents are n-hexane or toluene. Especially hexane has some advantages, such as it is less toxic than other solvents and does not form emulsions as toluene does **(Fletouris et al., 1998)**. The extraction with hexane was performed three times due to increased efficiency, as described **Oh, Shin and Chang (2001)**. Based on these authors, the chromatogram of method, which used hexane as the extraction solvent, had an excellent baseline and no interference was detected. The efficiency of extraction with hexane is also influenced by the presence of water **(Fletouris et al., 1998)**. Therefore, a small amount of water was added to the extraction solvent. The water was then removed by the filtration through anhydrous sodium sulphate. Almost the same steps were also described by **Borkovcová et al. (2009)**.

Based on these modified methods the cholesterol content in milk was analyzed by both techniques. By HPLC the mean content of cholesterol in milk was determined on 92.78 ±9.57 mg.kg^{-1} and by spectrophotometric determination on 84.57 ±10.95 mg.kg^{-1}. The 3D record of cholesterol peak in the milk sample is showed in Figure 3.

Ramalho, Casal and Oliveira (2011) determined the mean content of cholesterol in commercial milk samples on 11.6 ±0.2 mg.100 mL^{-1} by HPLC. According to **Faye et al. (2015)**, the mean values of cholesterol in cow milk are 8.51 ±9.07 mg.100 g^{-1}, which is close to our results.

From the results of **Manzi, Di Costanzo and Mattera (2013)**, the average cholesterol content in Italian cow´s milk is 12.8 ±0.4 mg.100 g^{-1}. Thus, on average, the cholesterol content of whole milk is 12 mg.100 g^{-1}. The variations of values can be attributed to variations in the processing of the milk as well as to differences in the animal breeds, individual characteristics, and intervals between milking, lactation phase, the composition of the animal´s diet, etc. **(Bauer et al., 2014)**.

To obtain the validation parameters, the linearity of both methods was performed by the calibration curves. The linearity is the ability of a method to demonstrate that its results are directly proportional to the concentration of the analyte in the sample, within the linear working range **(Ribeiro and Brandäo, 2017)**. In spectrophotometric determination, the linear range was obtained in the range of cholesterol content 0.1 to 1 mg with the correlation coefficient of 0.9992. In HPLC the linear range was achieved at the cholesterol concentrations at 25 to 350 mg.L^{-1} with the correlation coefficient at 0.9999. This result agrees with **Albuquerque et al. (2016)**, where the linearity was obtained over the range of 0.07-0.4 mg.mL^{-1}. The obtained LOD and LOQ for HPLC were 2.13 mg.kg^{-1} and 6.45 mg.kg^{-1}, respectively, while for the spectrophotometric method were 12.55 and 38.04 mg.kg^{-1}. Thus, from the results, it can be stated that HPLC has better sensitivity than spectrophotometric determination. The almost similar values of LOD and LOQ are reported by **Ahn et al. (2012)** with LOD 2.27 mg.kg^{-1} and LOQ 7.56 mg.kg^{-1}. The other important validation parameters are accuracy and precision.
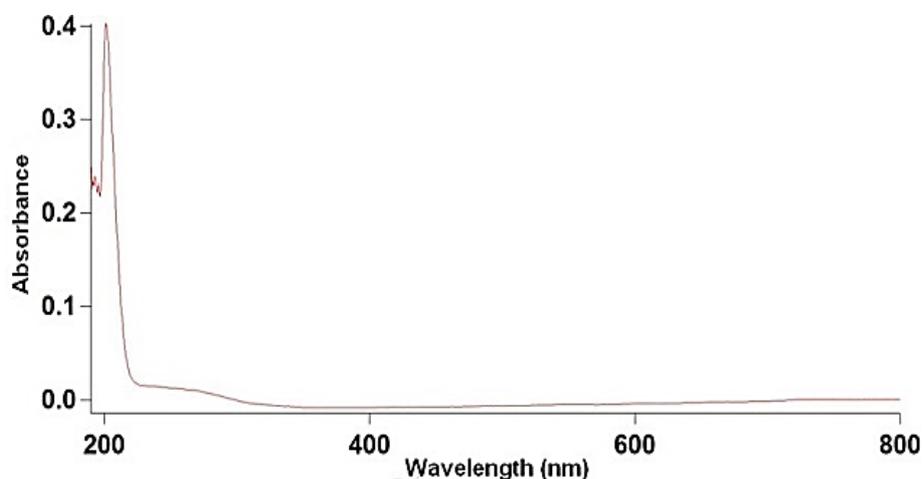

Figure 2 Absorbance spectrum of cholesterol in methanol obtained by UV-VIS spectrophotometer.
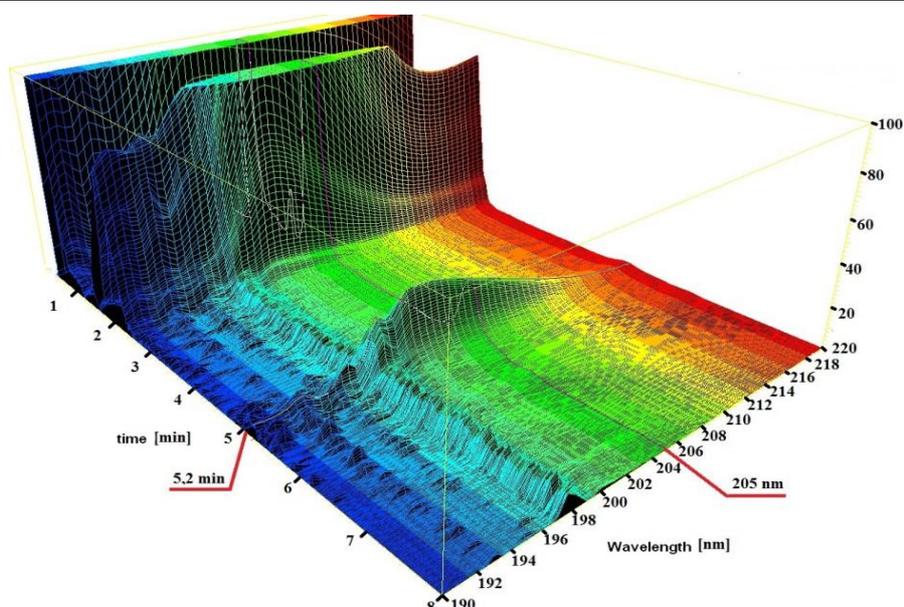
Figure 3 The 3D record of cholesterol peak in milk sample.

Table 1 Comparison of the results obtained from the analysis of cholesterol content in milk by HPLC (Method A) and spectrophotometric determination (Method B).

	Method A[a]	Method B[b]
Cholesterol content (mg.kg^{-1} ±SD)	92.78 ±9.57	84.57 ±10.95
LOD (mg.kg^{-1})	2.13	12.55
LOQ (mg.kg^{-1})	6.45	38.04
Recoveries (%)	91.05	85.34
Horrat	1.3	1.45
Slope of calibration curve (b)	433	0.558
Correlation coefficient (R[2])	0.9999	0.9992

Note: [a]n = 6, [b]n = 3, LOD – limit of detection, LOQ – limit of quantification.

The precision refers to the degree of agreement among repeated measurements. Precision is approved when Horrat parameter is less or equal 2 (**Ribeiro and Brandão, 2017**). Both methods showed good precision with Horrat value less than 2. Accuracy was obtained by the standard addition method at one concentration level of cholesterol. The recoveries were 85.34% and 91.05% for spectrophotometric determination and HPLC, respectively. Analyte recoveries close to 100% are ideal, but smaller values are admitted if the precision is good (**Bauer et al., 2014**), and in this case, it is proved by the Horrat values. The selectivity of chromatographic method was proven by the adequate separation of cholesterol with good resolution of the peaks and without co-elution of other compounds in the sample.

The comparison of the propose methods

The comparison of the results obtained from the analysis of cholesterol content in milk by HPLC and spectrophotometric determination is shown in Table 1. For the testing of conformity of the results obtained from both methods, Moore´s test was used according to **Eckschlager, Horsák and Kodejš (1980)**. Based on Moore´s test, the difference between cholesterol content in milk by HPLC and spectrophotometric determination is statistically insignificant at $p <0.05$ and the null hypothesis of consistency of results is accepted. The resulting mean cholesterol contents in milk determined by these two methods are thus relatively identical. The results showed an 8.8% difference. The cholesterol level in milk can be thus determined by either HPLC or spectrophotometric method. The same conclusion is described by **Essaka (2007)**. Based on his research, the agreement of the values obtained by HPLC and LB reaction with a 16% difference showed that the proposed method was indeed reliable.

As seen from validation parametres, HPLC has some advantages over spectrophotometry. Firstly, LOD and LOQ values are lower thus HPLC is more sensitive. Better sensitivity of HPLC can be seen also from the slope of the calibration curve, where the value is much higher than in spectrophotometric determination. The recoveries were lower in spectrophotometric determination, which can be caused by the different approaches in sample preparation. After saponification and extraction, the sample before spectrophotometry must be reacted with LB reagent, which could lower the recovery. Besides that, the color stability, the issue of temperature dependency, and the turbidity of the final color-developed solution have made colorimetric methods subject to significant concern regarding accuracy (**Dinh et al., 2011**). According to **Osman and Chin (2006)** HPLC was considered as the method of choice for

cholesterol determination with the lowest LOD and LOQ compare to spectrophotometry and gas chromatography. The performance of spectrophotometer was better than gas chromatography in terms of reproducibility.

CONCLUSION

This study was focused on the comparison of HPLC and spectrophotometric determination of cholesterol content in milk. From the results, the following conclusions can be postulated:

1. The spectrophotometric determination is influenced by the stability and absorbance characteristics of LB reagent.
2. The results obtained from HPLC and spectrophotometric determination differed only in 8.8% thus both methods are suitable for analysis of cholesterol in milk products.
3. HPLC analysis has some advantages over spectrophotometry, mainly higher sensitivity and lower LOD and LOQ values, which makes it more favorable in cholesterol determination in milk.

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