THE ACCURACY AND REPEATABILITY OF UV-DAD-HPLC METHOD FOR DETERMINATION OF CHOLESTEROL CONTENT IN MILK

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ABSTRACT
This paper is a report on the determination of the accuracy and repeatability of the proposed UV-DAD-HPLC method for the analysis of cholesterol content in milk sample. The accuracy and repeatability of the method are important values in the validation process and refer to the suitability of the method for given purpose. The sample preparation consisted of the saponification and extraction process. The conditions of saponification were as follows: time of saponification 15 min, the concentration of methanolic KOH solution 1 mol/L with a volume of 12 mL. The extraction of unsaponified residue was done two times with the mixture of n-hexane:chloroform (1:1, v:v). The samples were analyzed by HPLC chromatography with UV-DAD detector, C_{18} stationary phase, and with mobile phase consisted of an isocratic mixture of acetonitrile:methanol (60:40, v:v). In these conditions, the accuracy of method varied from 97.3 to 105.8%. The repeatability referred to the precision was evaluated from the relative standard deviation with the values ranging from 1.34 to 2.37%. From the results, it can be concluded that proposed HPLC method showed great accuracy and repeatability, and thus it could be suitable for the appropriate determination of cholesterol content in milk samples.
Keywords: cholesterol, HPLC, accuracy, repeatability, milk

Introduction

Cholesterol (3β)-cholest-5-en-3-ol) is a sterol lipid produced by animal cells. Most cholesterol is produced in liver, adrenal glands, intestines, and in gonads, whereas 20-25% of cholesterol comes from the diet of animal origin (Li et al., 2019). The word cholesterol may quickly be associated with chronic heart disease and other heart problems. However, it has also essential functions in the body such as providing essential components of membrane and serving as a precursor of bile acids, steroid hormones, and vitamin D (Daksha et al., 2010). As elevated human plasma cholesterol concentration may increase the risk of cardiovascular disease and atherosclerosis, a maximum intake of 300 mg per day for adults is recommended (Bertolín et al., 2018). From a nutritional point of view, cholesterol is not found in significant amounts in plant sources, is mostly present in foods of animal origin such as milk products, eggs, meat, and fish. Regarding its relationship with cardiovascular disease, the analytical methods for cholesterol evaluation in foods are crucial (Albuquerque et al., 2016). Cholesterol determination in foods usually involves lipid extraction, separation of cholesterol from interfering components or liberation of cholesterol into the free form, and measurement of isolated cholesterol. Cholesterol can be measured using gravimetry, titration, colorimetry, refractometry, fluorometry, and chromatography (Dinh et al., 2011; Kolarič and Šimko, 2020). Classical chemical methods are relatively simple and inexpensive to perform but multi-step procedures are required. Enzymatic assays involve the use of costly enzymes, although limits of detection are usually low. Chromatographic and mass spectrometric methods are the most accurate and sensitive (Li et al., 2019). The HPLC system equipped with either a UV/VIS detector or a photodiode array detector has been the most common alternative to gas chromatography for the analysis of cholesterol and other sterols in foods. The UV wavelengths for cholesterol absorption range from 203 to 214 nm with maximum absorbance at 205 nm because of the unsaturated center and the hydroxyl group (Dinh et al., 2011). According to Albuquerque et al. (2016), the reversed-phase HPLC coupled with UV or DAD detector is the most common technique for the evaluation of cholesterol content in foods. In our previous study (Kolarič and Šimko, 2020), it has been shown that both spectrophotometric and HPLC methods could be suitable for the determination of cholesterol content in milk but HPLC exhibited higher sensitivity and lower limits of detection. According to Osman and Chin (2006), the HPLC method was found to be the most convenient and consistent in giving highest sensitivity and accuracy for the determination of cholesterol. The propose of this work was thus the evaluation of the accuracy and repeatability of the proposed UV-DAD-HPLC method for the determination of cholesterol content in milk as these parameters are required for the appropriate method validation.

Materials and Methods

All reagents and standards were of analytical grade. Cholesterol standard was from Sigma-Aldrich with a purity ≥99%. Chloroform, n-hexane, ethanol, and sodium sulphate anhydrous were purchased from Centralchem s.r.o. (Bratislava, Slovakia). Methanol and acetonitrile (HPLC grade) were purchased from Fisher Chemical (Loughborough, UK). The cow´s milk (3.5% fat, Rajo a.s., Bratislava, Slovakia) was bought in a local market.

The saponification process was performed according to our previous study with slight modifications (Kolarič and Šimko, 2020). The 5.0 g of milk sample was refluxed with the methanolic solution of KOH (1 mol/L) using the volume of 12 mL and reaction time 15 min. The extraction process was performed two times with a mixture of n-hexane and chloroform (1:1, v/v)). The volume of extraction solvent was 15 mL. The extraction system consisted also of 10 mL of deionised water and 1 mL of ethanol (96%). After the extraction, the solvent was filtrated through anhydrous sodium sulphate, and evaporated using a vacuum evaporator (Heidolph, Germany). The residue was dissolved in 3 mL of methanol. The solution was filtered using syringe filters with PTFE membrane and particle size 0.2 µm (PTFE, 13 mm, 0.2 µm, Agilent Technologies, USA). The prepared solution was directly analysed by HPLC chromatography.

HPLC 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 0.5 mL/min using the mobile phase consisted of acetonitrile/methanol 60:40 (v/v). The injection volume was 10 µL and the temperature was set at 30 °C. As a stationary phase, a Zorbax Eclipse Plus C_{18} column (2.1x100 mm, 3.5 µm particle size, Agilent, USA) was used with the guard column Zorbax SB-C_{18} (4.6x12.5 mm, 5 µm particle size, Agilent, USA). Total run time of analysis was 7 min with retention time of cholesterol in 5.6 min. The results were recorded using the OpenLab CDS software, ChemStation Edition for LC, and LC/MS systems (product version A.01.08.108).

The accuracy of the method was determined by recovery tests after spiking the milk samples with cholesterol standard using five different concentrations (0.1, 0.3, 0.5, 1.0, and 1.5 mg/mL). After the quantification of the analytes in the fortified samples and in the control, the recovery percentage (% REC) was calculated according to equation 1 (Kolarič and Šimko, 2020):

$$\%REC = \left(\frac{Obtained\ conc. - Control\ conc.}{Expected\ conc.}\right) x\ 100 \quad (1)$$

The repeatability was investigated by injecting four replicates of sample in quadruple on the same day. The intermediate precision was evaluated on three different days by preparing four replicates from the same sample on each day. The precision was then evaluated from the relative standard deviation (RSD).

Statistical analysis was performed using Microsoft Excel version 365 and the results are expressed as mean ±standard deviation or as percentage.

Results and Discussion

Method validation is the process of defining an analytical requirement and confirming that the method under consideration has capabilities consistent with what the application requires. The performance characteristics commonly evaluated during method validation are selectivity, limit of detection and quantification, working range, analytical sensitivity, trueness (bias, recovery), precision (repeatability, intermediate precision, and reproducibility), measurement uncertainty, and ruggedness (Eurachem Guide, 2014). The accuracy of the method is determined as recovery and precision. In the cholesterol analysis in food products, accuracy is an important parameter as the sample preparation could vary in different food matrices.

The recovery of our proposed HPLC method for the determination of cholesterol content in milk is summarized in Table 1. The chromatograms of the analysis of spiked milk samples with the cholesterol standard at different concentrations are shown in Figure 1. Three general approaches are applicable for the evaluation of method recovery: 1. analysis of reference materials, 2. recovery experiments using spiked samples, and 3. comparison with the results obtained with another method (Eurachem Guide, 2014). In our study, the recovery of the method was studied by spiking samples with the cholesterol standard using five different concentrations (0.1, 0.3, 0.5, 1.0, and 1.5 mg/mL). The method showed great recovery ranging from 97.3 to 105.8%. The obtained results thus prove the efficiency of the proposed method. According to Bauer et. al (2014), the analyte recoveries close to 100% are ideal, but smaller values are admitted if the precision is good. Based on their results, the recovery of the method of cholesterol determination in milk ranged from 100.63 to 103.90% using the three different cholesterol standard concentrations (75, 150, and 300 µg/mL, respectively). Almost the same results were described by Ramalho et al. (2011). Their HPLC-DAD method for determination of cholesterol content in milk samples showed recovery ranging from 97 to 101%. The greater recoveries are described by Albuquerque et al. (2016), which varied from 111 to 125%. The recovery of the HPLC method is mainly influenced by two critical steps in sample preparation, the saponification and extraction. In the extraction process, the choice of extraction solvent is crucial. Most of the previous studies showed great recovery with the n-hexane solution. In our previous study (Kolarič and Šimko, 2020), the recovery using the pure n-hexane was only 91.05%. In this study, it was shown that the extraction with a mixture of n-hexane and chloroform (1:1, v/v) achieved better recoveries. Oh et al. (2001) studied the cholesterol

recovery using the different extraction methods (method A – saponification and extraction with diethyl ether, method B – extracted sample from method A passed through silica Sep-pak, method C – saponification and extraction with hexane, and method D – solid phase extraction) by adding 1.0, 2.0, and 3.0 mg of cholesterol to milk samples. Based on their results, the mean recoveries of methods A, B, C, and D were 99.3-100.2, 98.8-99.3, 100.1-100.3, and 99.7-100.7%, respectively, thus the differences were not significant. The saponification of the sample can be direct or indirect. The indirect saponification involves fat extraction, however, cholesterol forms complexes with other phospholipids and proteins, and this changes their overall physical properties, resulting in inefficient extraction of cholesterol, which explains the poor recovery of cholesterol (Oh et al., 2001). The direct saponification is thus preferred. In our study, the direct saponification with the methanolic KOH solution was also applied. Ahn et al. (2012) used method, which required only a sample pretreatment time of 5 min per sample and involved no-heating saponification. In the recovery test, the values were also satisfying, ranged from 98.11 to 102.34%. The recovery of the method of cholesterol determination in food is also influenced by the proper analytical technique. In our previous study, better recoveries were achieved by HPLC chromatography (91.05%) than spectrophotometric determination (85.34%). According to Albuquerque et al. (2016), the UHPLC method recovery ranged from 80 to 106% in comparison to HPLC (111 to 125%). Osman and Chin (2006) described that the mean recoveries of methods using a spectrophotometer, HPLC, and gas chromatography were 86.67-126.67, 73.33-110.00, and 60.00-146.67%, respectively.

Table 1: The recovery of proposed HPLC-UV-DAD method for the determination of cholesterol content in milk.

Added cholesterol standard concentration [mg/mL]	The cholesterol content in milk sample [mg/kg] [a]	Recovery [%]	RSD [%]
0.0	105.78 ±1.35	-	-
0.1	104.51 ±2.20	98.8	2.11
0.3	111.89 ±0.32	105.8	0.29
0.5	111.17 ±0.96	105.1	0.87
1.0	102.93 ±1.25	97.3	1.22
1.5	104.16 ±3.36	98.5	3.23

[a] The values are expressed as mean ±standard deviation; RSD, relative standard deviation

The repeatability is a measure of the variability in results when a measurement is performed by a single analyst using the same equipment over a short timescale while intermediate precision gives an estimate of the variation in results when measurements are made under more variable conditions (Eurachem Guide, 2014). In this study, the repeatability and intermediate precision were determined comparing the standard deviation (SD) and relative standard deviation (RSD) of the results obtained in three days. The repeatability refers to precision, which is important for the final accuracy of the proposed method. The values obtained for the repeatability and intermediate precision of the proposed method for cholesterol determination in milk are found in Table 2. From the results, it can be noticed that our method showed great repeatability with the RSD varied from 1.3 to 2.8% and intermediate precision with the RSD 0.5% thus our method is precise. The RSD of 3% was described by Ramalho et al. (2011) for intermediate precision while Albuquerque et al. (2016) showed the RSD values ranging from 1.66 to 1.95%. The RSD values of up to 15% are acceptable, although a maximum variation of 5% for micro constituents is recommended (Bauer et al., 2014). In general, the HPLC or gas chromatography has the highest precision in the analysis of cholesterol content in food, where the HPLC-UV seems to be the most suitable. The comparison of different methods for the determination of cholesterol content in food samples described by Daneshfar et al. (2009) showed that the RSD values obtained by electrophoresis were up to 6.3%, reverse micelle up to 11%, solid phase extraction-gas chromatography-flame ionization up to 3.6%, and HPLC-fluorimetric up to 5.6% while HPLC-UV showed the RSD 3.1%. Higher interday precision using gas chromatography tandem mass spectroscopy in the analysis of cholesterol in milk powder was also noticed by Chen et al. (2015) with RSD 8%.

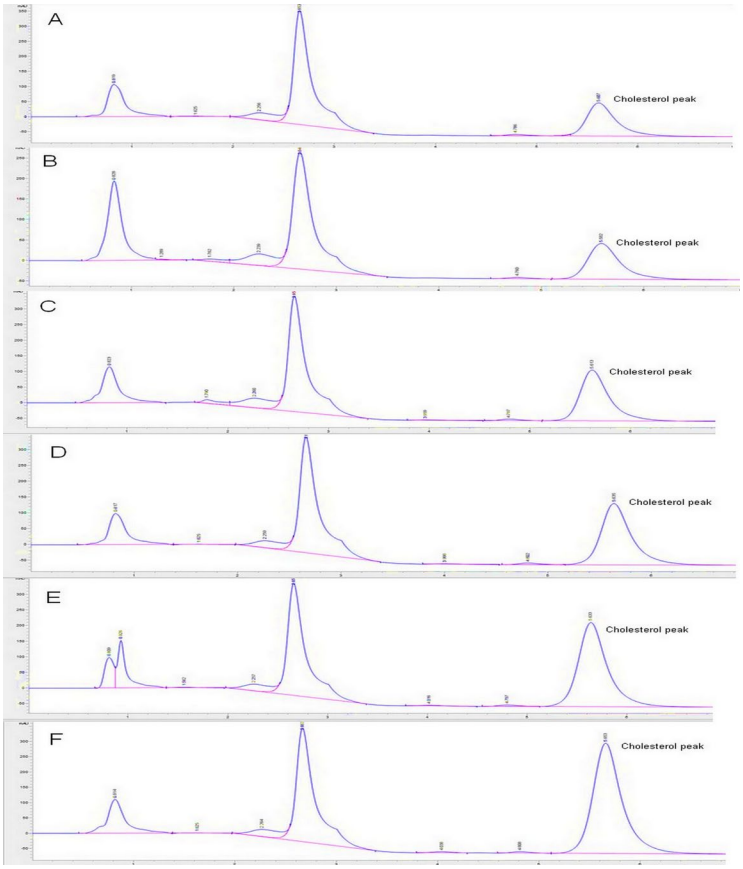

Figure 1. The chromatograms of the analysis of spiked milk samples with the cholesterol standard at different concentrations: A – control sample, B – 0.1 mg/mL, C – 0.3 mg/mL, D – 0.5 mg/mL, E – 1.0 mg/mL, and F – 1.5 mg/mL.

Table 2: The repeatability and intermediate precision of the proposed method for cholesterol determination in milk

Repeatability	The cholesterol content in milk sample [mg/kg] [a]	RSD [%]	Intermediate precision RSD [%]
Day 1	120.92 ±2.86	2.37	0.53
Day 2	120.47 ±1.61	1.34	
Day 3	119.41 ±2.37	1.98	

[a] The values are expressed as mean ±standard deviation; RSD, relative standard deviation

Conclusion

In this study, it was proven that proposed UV-DAD-HPLC method for the determination of cholesterol content in milk has great accuracy, precision, and repeatability. The sample preparation was optimized by using the extraction solvent consisted of the mixture of n-hexane:chloroform (1:1, v:v), and the saponification time in 15 min. In these conditions, the recovery of method varied from 97.3 to 105.8%, and the repeatability referred to the precision from 1.3 to 2.4%. These findings are important as the solvent consumption and time of sample preparation was optimized and simplified in comparison to the previously published HPLC methods. This method is thus accurate and can be used for appropriate evaluation of cholesterol content in milk.

Acknowledgements

This work is the result of the project implementation "Building Infrastructure for Modern Research of Civilization's Diseases" (ITMS 26230120009) financially supported by the Research & Development Operational Programme funded by the ERDF, grant APVV- 061-2018, and grant VEGA 1/0583/20.

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