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Determination of cholesterol content in foods and functional foods by gas chromatography mass spectrometry GC-MS

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Abstract

Cholesterol is a soft, pale yellow steroid fat that exists in the body's cell membranes and is transported in the plasma of most animals. This compound plays an important role in the physiology of the human body. However, excess cholesterol in the diet is also one of the leading causes of cardiovascular diseases. Therefore, the quantification of cholesterol in foods and functional foods is essential so that consumers can choose a diet containing the right amount of cholesterol. This study used gas chromatography-mass spectrometry to quantify cholesterol, with ISQ 7000 GC-MS system, TraceGOLD TG-5MS chromatographic column, and electron impact (EI) ionization mode. The validity of the method was confirmed according to the guidelines of the Association of Official Analytical Collaborations (AOAC). The results showed that the method has good specificity, the linearity range from 5 to 100 µg/mL in working solution, the detection limit of 1.5 to 15 mg/kg, the quantification limit of 5.0 to 50 mg/kg, depending on the matrix; the method's recovery were from 92.6 to 108.4% and the precision has relative standard deviations of 1.18 - 2.84%, meeting the requirements of AOAC. The method was used to analyze samples of dairy foods and functional foods randomly purchased in the market.

Keywords: *Cholesterol, GC-MS, dairy-based foods, functional foods.*

1. INTRODUCTION

Cholesterol is a soft, pale yellow steroid fat (Figure 1). Cholesterol is present in the cell membranes of all tissues in the body. It is a component that makes up the cell membrane and helps stabilize the cell membrane structure. Cholesterol is also the raw material for synthesizing bile salts, vitamin D and steroid hormones, including salt-water regulating hormones and sex hormones [1]. Besides its extremely important physiological roles, cholesterol is also a leading cause of cardiovascular diseases. Too much cholesterol in the diet can increase the amount of low-density lipoprotein cholesterol in the blood (LDL-cholesterol). LDL-cholesterol is the main cause of atherosclerosis [2], which in turn leads to many other cardiovascular diseases such as hypertension or myocardial infarction. Excess

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cholesterol comes from diets containing a lot of foods derived from meat, eggs, and animal milk - foods rich in cholesterol. According to Recommendations on the diagnosis and treatment of lipid disorders in 2015 by the Vietnam National Heart Association, cholesterol in the daily diet should be below 300 mg to control cardiovascular risk [3]. Therefore, quantifying cholesterol in food is very important to help consumers choose foods containing the appropriate amount of cholesterol and avoid loading too much cholesterol into the body.

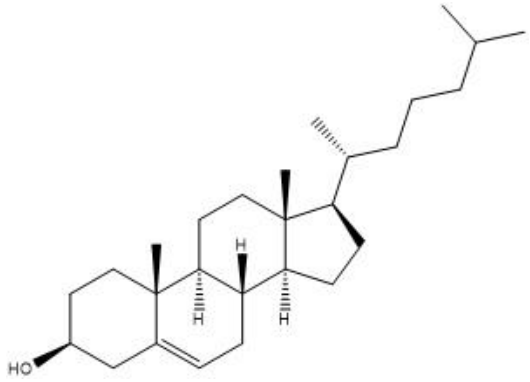

Figure 1. *Chemical structure of cholesterol*

In the world, there are many methods for quantifying cholesterol using gas chromatography, in Vietnam there is also the TCVN method for determining cholesterol in food [4-6]. These methods all have the process of treating samples by direct saponification and then extracting cholesterol in the saponification solution with organic solvents. However, these methods often use direct extract for analysis [4, 5], or do not create ether derivatives for cholesterol before gas chromatography analysis [6]. This can result in a high background signal in the working solution, containing many impurities, or a poor signal of cholesterol in the analysis. The cholesterol quantification method in TCVN overcomes the above disadvantages, however, the sample processing process uses toluene as the extracting solvent [7] which easily creates an emulsion, making the process difficult and time-consuming. Therefore, in this study, the gas chromatography method was chosen with a sample treatment process that was changed in terms of saponification conditions and extracting solvent to optimize the conditions of gas chromatography to analyze cholesterol in foods and functional foods samples. Among them, dairy foods and dairy functional foods are common ingredients in the daily diet. Therefore, this study focuses on analyzing types of milk in Hanoi to preliminarily assess the cholesterol content in types of milk used daily, thereby serving as a basis to help consumers evaluate and choose types of suitable milk.

2. MATERIALS AND METHODS

2.1. Research subjects

The subject of this study is cholesterol in foods and functional foods derived from milk (animal or vegetable), in both powdered and liquid milk forms, randomly purchased at markets, stores, and supermarkets in Hanoi.

2.2. Chemicals and reagents

Cholesterol with 99% purity was purchased from Sigma (Batch number SLBW6939). 5α-cholestane with 99% purity was purchased from Sigma (Lot number 126M4089V). Merck's solvents and chemicals include ethanol, potassium hydroxide, sodium chloride, n-hexane, dimethylformamide (DMF), toluene, petroleum ether, hexamethyldisilazane (HMDS), chlorotrimethylsilane (TMCS), bis(trimethylsilyl)trifluoroacetamide (BSTFA), deionized water.

2.3. Instruments

Thermo Scientific's ISQ 7000 GC-MS system includes: Trace 1310 gas chromatography system, ISQ 7000 single quadrupole mass spectrometer ExtractaBrite ion source, CombiPAL CTC Analytics automatic sample injector AG integrates vapor headspace sampling; TraceGOLD TG-5MS chromatography column from Thermo Scientific: column length 30 m, inner diameter 0.25 mm, stationary phase film thickness 0.25 µm.

Some other auxiliary equipment includes analytical balance (accuracy 0.1 mg) XS105 (Mettler Toledo); Mikro 200R cold centrifuge (Hettich); vertical shaker SA300 (Yamato); rotary vacuum evaporator (Eyela - Japan) and other common equipment and tools in the laboratory.

2.4. Methods

2.4.1. Conditions for cholesterol analysis by gas chromatography-mass spectrometry (GC-MS)

Conditions for running chromatography and mass spectrometry were referred to Chen et al. [6]. Specific GC temperature program (Figure 2): sample inlet held at 250°C, column heated at 50°C held for 1 minute; increase heat 50°C /min to 100°C, hold for 2 minutes; increase heat 50°C /min to 270°C, hold for 20 minutes; increase heat 50°C /min to 300°C, hold for 2 minutes; helium carrier gas at a rate of 1 mL/min. Specific MS conditions: path temperature from GC to MS section kept at 280°C, ion source temperature at 250°C, electron impact ionization (EI) mode, 70 eV.

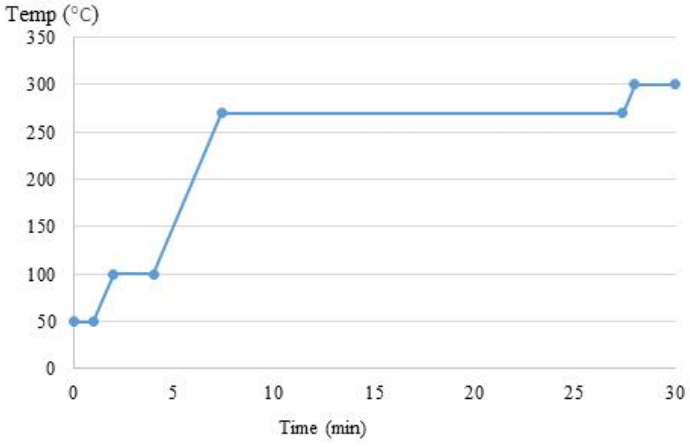

Figure 2. *GC temperature program*

2.4.2. Optimizing sample preparation conditions

Based on references [6, 7], the sample processing procedure was selected as follows: each sample was accurately weighed an appropriate amount into a 250 mL conical flask, for powder samples, add about 5 mL of distilled water and shake well to wet the sample. After that, 50 mL of KOH solution in 96% ethanol and 100 µL of 100 µg/mL 5α-cholestane internal standard solution in n-hexane was added. The jar was closed and incubated in the oven at 80°C for 120 minutes. After cooling the flask to room temperature, the entire solution in the flask was transferred to a 500 mL separatory funnel. The reaction flask was rinsed with about 20 mL of water and 10 mL of 96% ethanol. The washing solution and the saponification solution were combined. The mixture was shaken with 150 mL n-hexane on a vertical shaker at level 9 for 30 minutes. After layering, the lower layer of alcohol was removed, then about 100 mL of 20% NaCl solution in water was added into the funnel and shaken vigorously for about 20 seconds. After removing the lower water layer, the n-hexane solution was filtered through filter paper containing about 10 g of anhydrous Na_2SO_4 into a 250 mL flask. The liquid was then evaporated in the flask at 60°C until it dries. 10 mL of DMF was exactly added to the flask and shaken vigorously for 2 minutes to dissolve the residue, insoluble residue was filtered if needed. Exactly 1 mL of DMF solution was pipetted into a 15 mL centrifuge tube, and then 200 µL HMDS and 100 µL TMCS were added to the tube. After shaking well and letting the tube stand for 15 minutes, exactly 1 mL n-hexane and about 5 mL distilled water were added to the tube. The tube was closed and shaken well, then centrifuged for 1 minute at 6000 rpm. Finally, the n-hexane layer was transferred into a vial and analyzed by GC-MS. A sample of milk powder that has been determined to contain cholesterol content was used to optimize the process, specifically as follows:

- Optimizing concentration of KOH solution in 96% ethanol: 5%, 10%, 20%, 30% (m/v).
- Optimizing incubation temperature of the saponification vessel: room temperature (21°C), 40°C, 60°C, 80°C, 100°C.
- Optimizing saponification time: 15 minutes, 30 minutes, 60 minutes, 120 minutes and 180 minutes.
- Optimizing extracting solvents: n-hexane, toluene, petroleum ether, n-hexane: petroleum ether (1:1, v/v).
- Optimizing derivatization reagents: HMDS:TMCS (2:1, v/v), BSTFA:TMCS (99:1, v/v).

2.4.3. Method validation

The criteria evaluated to evaluate the method include specificity (IP score, ion ratio, analysis of blank sample, standard sample and spiked sample), limit of detection, limit of quantification (based on the signal-to-noise ratio S/N), linearity (build a standard curve from 5-100 µg/mL on the working solution), repeatability and recovery (perform the analysis in duplicate 6 times at 3 concentrations levels 5, 20 and 50 µg/mL on working solution). The results were evaluated and compared with regulations under AOAC 2016 [8] and EC 2021/808 [9].

3. RESULTS AND DISCUSSION

3.1. Conditions for cholesterol analysis by GC-MS

Based on the analytical conditions selected in section 2.4.1, a 10 µg/mL cholesterol standard solution was derivatived and analyzed, then determined the ions during the analysis process. The results are summarized in Table 1 (IP score calculated according to EC standard 2021/808 [9]).

Table 1. *Cholesterol's IP score*

Compound	***Ions***			***IP scores***
	Quantitation	***Qualification***		
Cholesterol	129.10	386.43	329.43	4

Based on the results, the IP score of cholesterol is 4, suitable for analysis on mass spectrometry (according to EC 2021/808 [9]). Thus, GC-MS conditions have achieved the necessary specificity to be used to optimize sample preparation and cholesterol analysis processes.

3.2. Optimization of the sample preparation process

The process was optimized under different conditions, each condition repeated 3 times. The results of each survey will be compared through the parameters:

$$A = \frac{S_{CHO}}{S_{5AC}} . \frac{1}{m} (1/g)$$

S_{CHO}: peak area of cholesterol.

S_{5AC}: peak area of 5α-cholestane.

m: sample weight (g).

3.2.1. Optimization of concentration of KOH solution in 96% ethanol

Samples were prepared with KOH solution in 96% ethanol at concentrations of 5%, 10%, 20%, 30% (m/v). The results are shown in Figure 3.

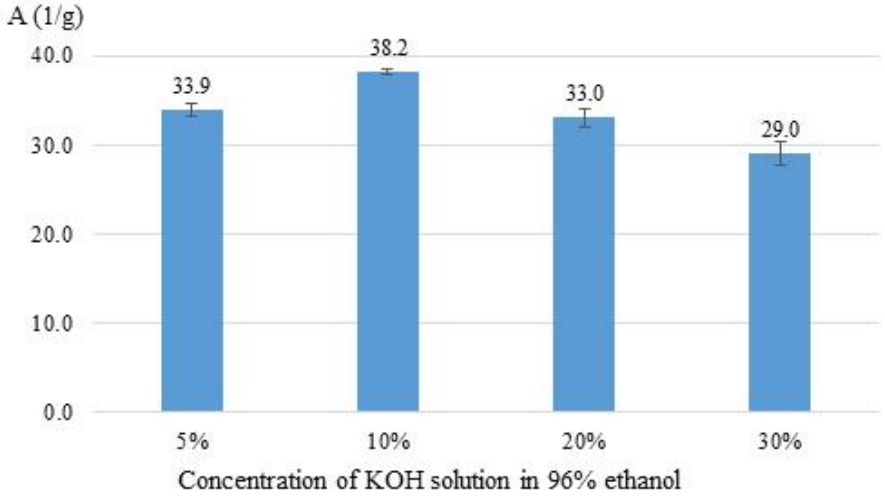

Figure 3. *Results of optimizing KOH concentration in 96% ethanol*

The results show that when using 10% KOH solution, the best results are obtained, the signal increases when increasing the concentration from 5% to 10%, and then when continuing to increase the concentration to 20% and 30%, the results gradually decrease. This can be explained as follows: when increasing the concentration of KOH solution from 5% to 10%, the increased amount of KOH helps the saponification reaction occur quickly and completely, releasing maximum cholesterol from bound forms. into free form; When continuing to increase the KOH concentration to 20% and 30%, the viscosity of the saponification solution increased, limiting the movement of molecules, and reducing the reaction rate, while the amount of KOH at a concentration of 10% was reduced. excess for the reaction to complete. Therefore, the research team chose 10% KOH solution to perform the reaction to get the best results.

3.2.2. Optimization of saponification reaction temperature

The process was optimized with different saponification reaction temperature levels: room temperature (21°C), 40°C, 60°C, 80°C, 100°C. The results are shown in Figure 4.

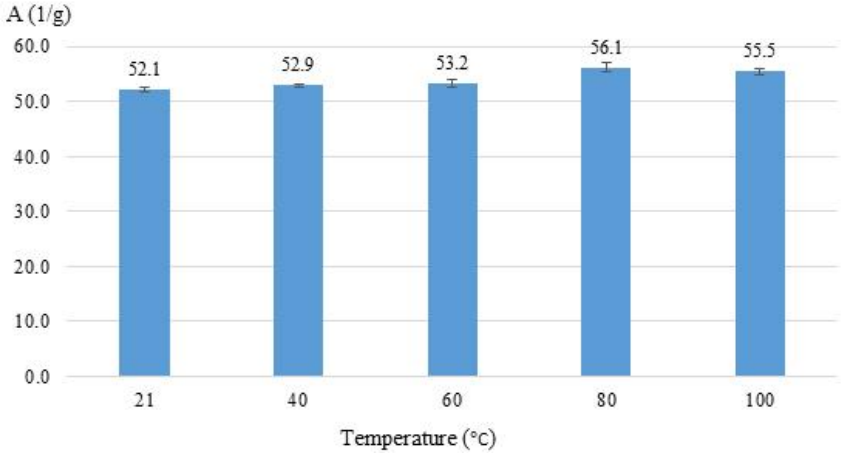

Figure 4. *Results of optimizing the incubation temperature of the saponification reaction vessel*

It can be seen that the signal gradually increases as the saponification temperature increases, because the higher the temperature, the faster the reaction occurs. However, after increasing to 80°C, if the temperature was increased to 100°C, the signal decreased. This phenomenon may occur because the saponification solution is mainly ethanol with a concentration of about 96% and a boiling temperature of about 80°C, If the temperature is increased too much above this temperature, the boiling ethanol will evaporate, increasing the viscosity of the solution slows down the reaction. Therefore, the saponification temperature was chosen at 80°C to obtain optimal analytical results.

3.2.3. Optimization of saponification time

A saponification reaction survey was conducted at intervals of 15 minutes, 30 minutes, 60 minutes, 90 minutes, and 120 minutes. The survey results are shown in Figure 5.

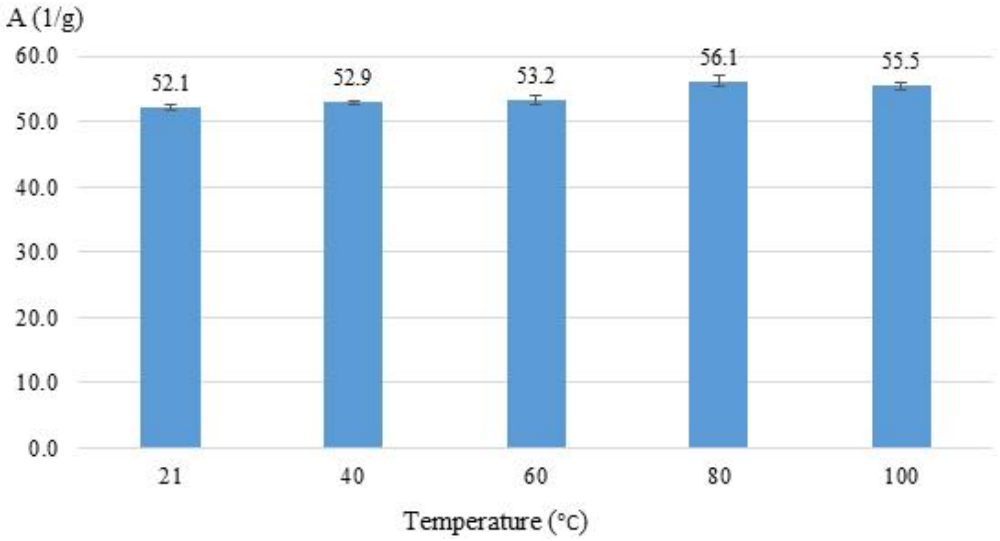

Figure 5. *Results of optimizing saponification time*

From the above results, we see that when increasing the saponification time to 60 minutes, the results obtained gradually increase because the longer the saponification time helps the reaction to occur more thoroughly. However, from 60 minutes, the signal does not change significantly, because 60 minutes is enough for the reaction to occur almost completely, continuing hydrolysis does not help increase the quantitative results. Therefore, the research team chose a saponification time of 60 minutes to both obtain good analysis results and save analysis time.

3.2.4. Optimization of extracting solvent

Samples were prepared with different extracting solvents: n-hexane, n-hexane: petroleum ether (1:1, v/v), petroleum ether, and toluene. The survey results are shown in Figure 6.

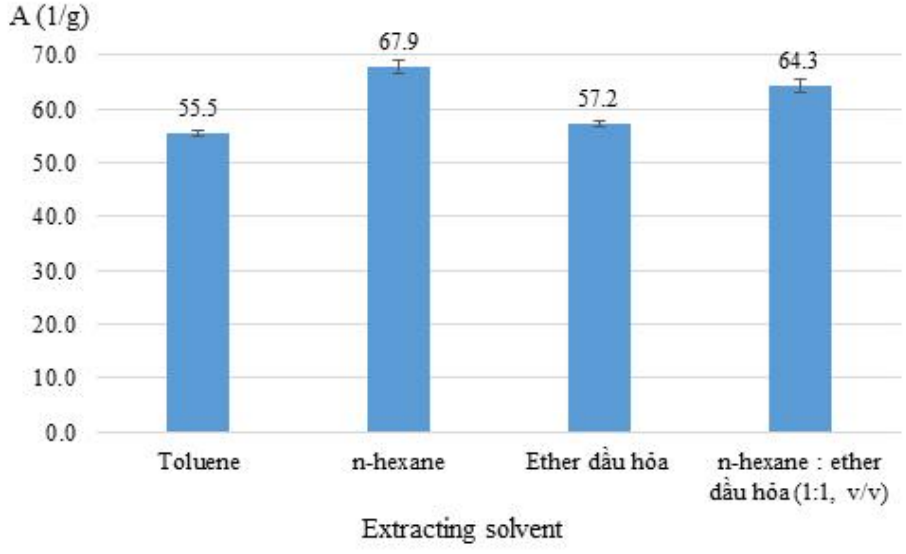

Figure 6. *Results of optimizing extracting solvent*

The results when extracting with n-hexane are the best. Petroleum ether does not give extraction efficiency as good as n-hexane, so the results gradually decrease when extracted with a mixture of n-hexane: petroleum ether ratio (1:1, v/v) and petroleum ether. Toluene solvent when shaken creates a strong emulsion, difficult to separate layers; When using ethanol to break the emulsion, a large amount is needed (the research team actually needed about 70 mL of ethanol to completely break the emulsion), the ethanol can dissolve with the toluene layer. At the same time, after evaporation, the toluene solution left many insoluble residues and poor analytical signals. N-hexane did not present the same difficulties as using toluene. For the above reasons, n-hexane was chosen as the optimal extraction solvent in this study.

3.2.5. Optimization of derivative reagents

Conduct a survey of the reagents used for derivatization as HMDS:TMCS (2:1, v/v), BSTFA:TMCS (99:1, v/v). Derivatives that produce a trimethylsilyl ether derivative of cholesterol assisted the analyte vaporize more easily, creating more favorable conditions for gas chromatography. The results of the survey are presented in Figure 7.

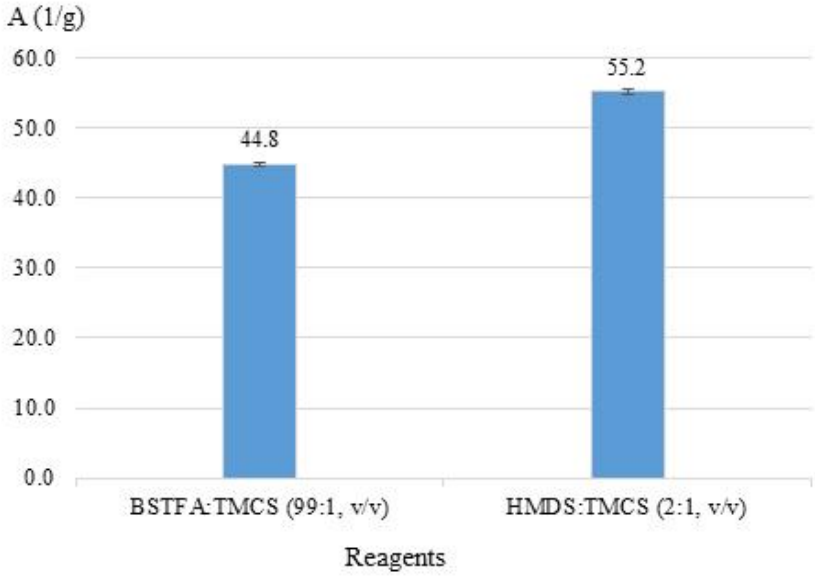

Figure 7. *Results of optimizing derivative reagents*

Survey results show that: at the same amount of derivative, cholesterol derivation with HMDS:TMCS (2:1, v/v) gives a better signal than BSTFA:TMCS (99:1, v/v). HMDS:TMCS (2:1, v/v) is the optimal derivative for this quantification procedure.

3.2.6. Optimal sample preparation process

After getting all the results of the survey, the optimal sample preparation process was presented in Figure 8. For the amount of sample to be weighed, remember to weigh an appropriate sample amount of about 0.5-1 g for powdered milk food samples; and about 2 - 10 g with liquid dairy foods.

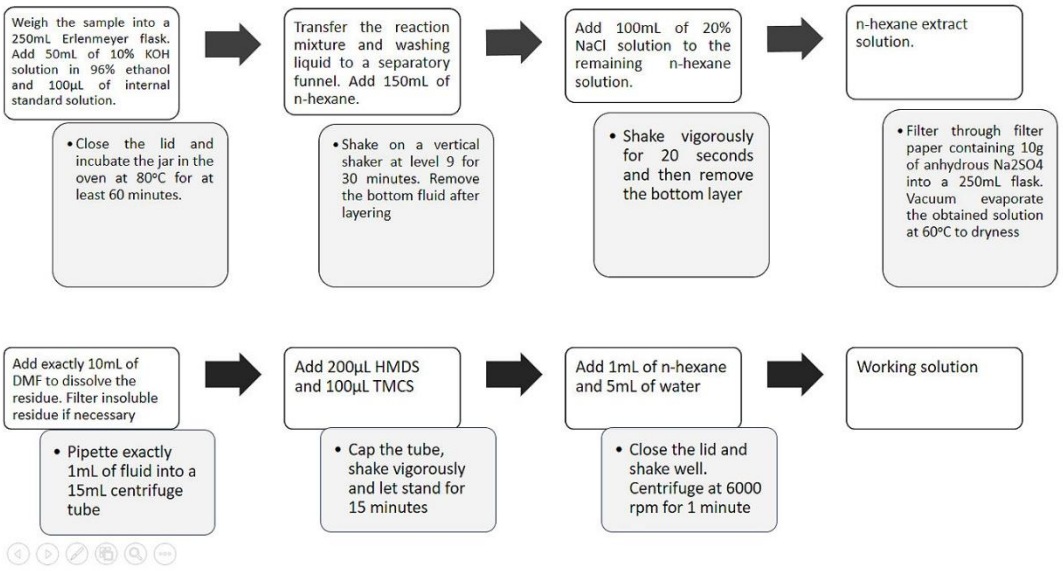

Figure 8. *Optimal sample preparation process*

3.3. Method validation

3.3.1. Specificity

The specificity of the method was evaluated by analyzing blank samples, standard samples, and spiked blank samples. The results of the specificity assessment chromatogram in Figure 9 show that the blank sample did not show any signal of the analyte, the spiked sample and the standard sample had signals at the same retention time, and the difference was no more than 2%. Thus, the method meets the requirements for specific validation parameters for cholesterol analysis (according to AOAC [8]).

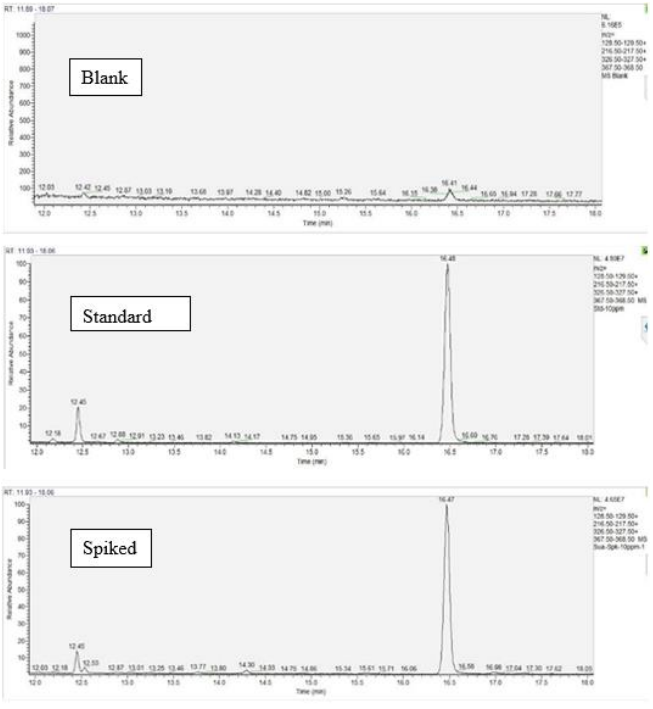

Figure 9. *Results of specificity assessment*

+ IP score: mass spectrometric analysis of cholesterol with 3 ions, IP score = 5 (according to section 3.1), meeting the requirements for mass spectrometric analysis (EC 2021/808 [9]).

+ Ion ratio: For mass spectrometry analysis method, ion ratio is the criterion to confirm the presence of analyte: conduct analysis on the standard sample and spiked sample, compare the intensity ratio ions obtained from 2 ions 329.43 and 129.10. The results are presented in Table 2.

Table 2. *Ion ratio of cholesterol*

	Standard	***Spiked***	R_{diff}	***Requirement***
Cholesterol	45.3%	45.7%	0.75%	± 15%

The results in Table 2 show that: the ion ratio of the spiked sample is within the maximum allowable error range, so the method meets the criteria for the ratio of ions in the standard and spiked samples (EC 2021/808 [9]).

3.3.2. Limit of detection (LOD) and limit of quantification (LOQ)

Spiked blank samples were prepared to obtain working solutions at concentration levels of 2, 5, and 10 µg/mL. The working solutions were then analyzed to determine LOQ values. The analyte signal-to-noise ratio (S/N) at the three concentration levels are 9, 12, 24 respectively (Figure 10). We see that this ratio at a concentration of 5 µg/mL is consistent with the LOQ concentration level, so the LOQ concentration is determined to be 5 µg/mL. From this, the LOD concentration is deduced to be 1.5 µg/mL. This concentration level in the working fluid corresponds to the cholesterol content in the sample as follows: LOD is 1.5 mg/kg with liquid milk matrix and 15 mg/kg with powdered milk matrix, LOQ is 5 mg/kg with liquid milk matrix and 50 mg/kg for powdered milk matrix.

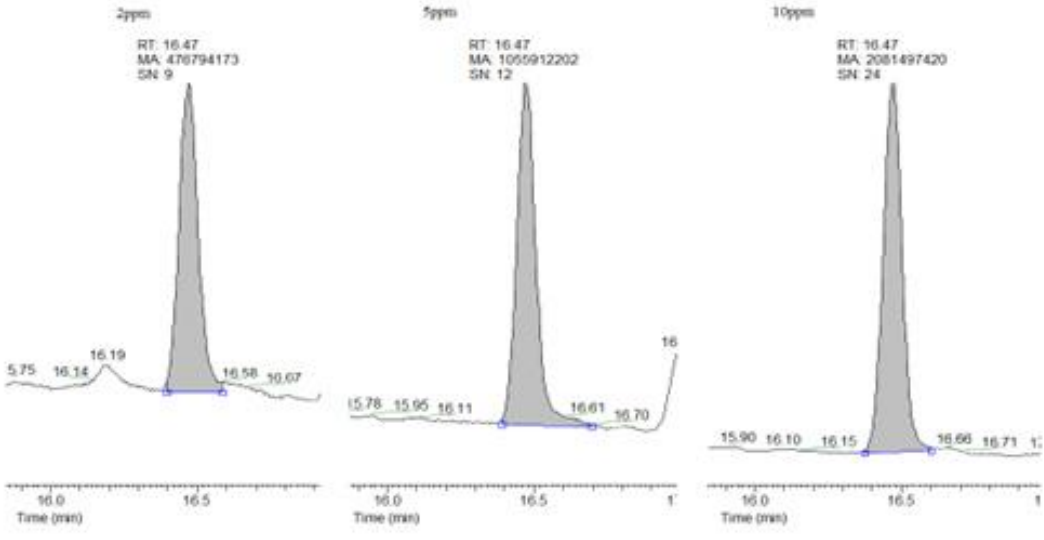

Figure 10. *Results of S/N ratio at different concentrations*

3.3.3. Calibration curve and linearity

Standard solutions in DMF at concentration levels of 5, 10, 20, 50 and 100 µg/mL were derivatived and analyed. Then we built a calibration curve between cholesterol concentration and peak area ratio of cholesterol and 5α- cholestane, the results are shown in Figure 11.

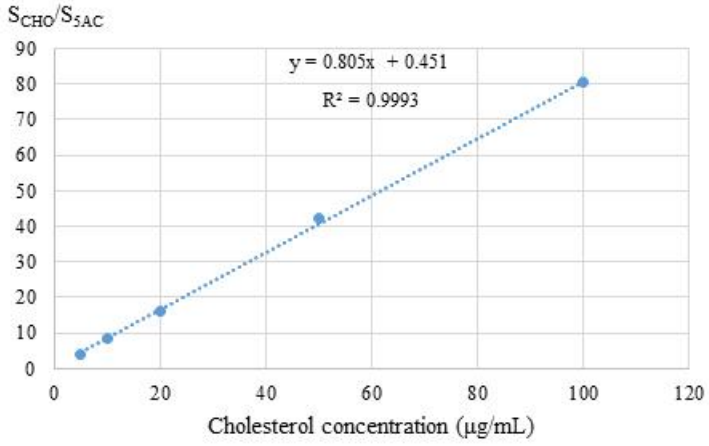

Figure 11. *Linearity assessment results*

From the above results, we can calculate the bias of the standard concentration levels, the results are shown in Table 3.

Table 3. *Bias of the standard concentration levels*

Actual concentration (µg/mL)	***Concentration calculated according to standard curve (µg/mL)***	***Bias***
5	4.6	-8.0%
10	10.0	0%
20	19.3	-3.5%
50	51.9	3.8%
100	99.2	-0.8%

The results show that the above standard curve has a coefficient of determination $R^2 >$ 0.99 and the deviation of the standard points is no more than 15%. This proves that the concentration range from 5 µg/mL to 100 µg/mL has a linear dependence between the ratio of analyte peak area and internal standard with analyte concentration.

3.3.4. Accuracy and precision

Spiked blank samples were analyzed to obtain working solutions at concentration levels: 5, 20 and 50 µg/mL. Each concentration level was analyzed 6 separate times. The 5 µg/mL level was analyzed for 2 days, 6 times a day to measure intermediate precision. The results obtained recovery at standard addition levels from 92.6 - 108.4%; Repeatability: 1.18 - 2.84%, intermediate precision 3.52 - 3.64% depending on the sample matrix. The recovery and precision of the method met the requirements of AOAC [8].

3.3.5. Application of cholesterol analysis in dairy foods and functional foods

Various food samples were analyzed, including liquid and powdered foods derived from nuts and animal milk purchased randomly on the market. The results of the analysis are presented in Table 4.

Table 4. *Cholesterol content in foods and functional foods*

No	***Sample***	***Cholesterol content (mg/kg)***
1	Soymilk	< LOQ
2	Roasted rice milk	< LOD
3	Walnut milk	< LOD
4	UHT milk	159.1
5	Formula drinking milk for diabetics (Medical nutritional food)	11.3
6	Orange flavored yogurt drink	47.0
7	Powdered milk (Nutritional supplements)	738.3
8	Powdered milk (Nutritional supplements)	353.5
9	Powdered milk (Nutritional supplements)	355.4
10	Powdered milk (Nutritional supplements)	738.4

Different dairy products have various cholesterol levels. Plant-based milk products contain virtually no cholesterol. Products made from animal milk often contain relatively high levels of cholesterol, especially nutritional supplements in the form of milk powder that contain very high cholesterol levels of 300 mg/kg. However, when mixed according to the formula printed on the package - about 40 g for 200 mL of milk - the content in each mL of milk (equivalent to about 1 g of milk) is only 60 mg/kg. With a daily consumption of about 100 g of powdered milk or 500 mL of liquid milk (equivalent to about 500 g), the amount of cholesterol from milk can account for more than 25% of the maximum amount according to Recommendations for Diagnosis and Treatment of Lipid Disorders. 2015 by the Vietnam National Cardiology Association [3]. This can be the basis to help consumers balance their menus as well as choose appropriate foods to proactively balance their daily nutrition. UHT milk samples of animal origin analyzed in this study had cholesterol levels similar to liquid milk samples of animal origin analyzed in previous studies, about 10.1 – 17.7 mg/100 g [4, 10, 11]. Plant milk contain no or very small amounts of cholesterol because cholesterol is an animal sterol. Choosing these types of milk to replace animal milk can be a way to limit cholesterol in your daily diet.

4. CONCLUSION

The study used the GC-MS technique to determine cholesterol content in foods and dairy functional foods. The analytical procedure includes: treating the sample by saponification in 10% KOH solution in ethanol, then extracting cholesterol with n-hexane and derivatizing the cholesterol into ether with HMDS:TMCS (2:1, v/v); The temperature gradient program and mass spectrometry conditions are selected appropriately. The method has been validated and the results meet the requirements of AOAC [8] and EC 2021/808 [9] (LOD was 1.5 mg/kg for liquid milk matrix and 15 mg/kg for milk matrix powder, LOQ was 5 mg/kg for liquid milk matrix and 50 mg/kg for powdered milk matrix, recovery 92.6 - 108.4% and repeatability 1.18 - 2.84%). In the 10 milk samples analyzed, the plant-based milk samples had almost no detectable cholesterol, while the animal-origin milk samples contained relatively high amounts of cholesterol. Research will continue to be expanded to analyze cholesterol content in other dairy products on the market.

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Xác định cholesterol trong thực phẩm, thực phẩm chức năng bằng sắc ký khí khối phổ (GC-MS)

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[2] Viện Kiểm nghiệm an toàn vệ sinh thực phẩm Quốc gia, Hà Nội, Việt Nam

Tóm tắt

Cholesterol là một chất béo steroid, thể chất mềm, màu vàng nhạt, tồn tại trong màng tế bào của cơ thể và được vận chuyển trong huyết tương của hầu hết các loại động vật. Hợp chất này có vai trò quan trọng trong sinh lý của cơ thể. Tuy nhiên, cholesterol dư thừa trong khẩu phần ăn cũng là một trong những nguyên nhân hàng đầu trong các bệnh lý về tim mạch. Vì vậy, việc định lượng cholesterol trong các loại thực phẩm, thực phẩm chức năng là rất cần thiết để người tiêu dùng có thể lựa chọn khẩu phần ăn chứa lượng cholesterol phù hợp. Nghiên cứu này sử dụng phương pháp sắc ký khí khối phổ để định lượng cholesterol, với hệ thống ISQ 7000 GC-MS, cột sắc ký TraceGOLD TG-5MS, chế độ ion hóa va chạm điện tử (EI). Giá trị sử dụng của phương pháp được xác nhận theo hướng dẫn của Hiệp hội hợp tác phân tích chính thức (AOAC). Kết quả cho thấy phương pháp có tính đặc hiệu tốt; đường chuẩn tuyến tính trong khoảng nồng độ 5-100 µg/mL của dung dịch làm việc; giới hạn phát hiện 1,5 mg/kg đến 15 mg/kg, giới hạn định lượng 5 mg/kg đến 50 mg/kg tùy theo nền mẫu; độ thu hồi của phương pháp từ 92,6 - 108,4% và độ chụm có độ lệch chuẩn tương đối 1,18 – 2,84%, đáp ứng theo yêu cầu của AOAC. Phương pháp đã được sử dụng để phân tích các mẫu thực phẩm, thực phẩm chức năng nguồn gốc từ sữa được mua ngẫu nhiên trên thị trường.

Từ khóa: *Cholesterol, GC-MS, thực phẩm và thực phẩm chức năng nguồn gốc từ sữa.*