Chapter 6

Cholesterol

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Abstract

The cholesterol determination in meat and meat products is very important because these products are highly demanded. The intake of high levels of cholesterol is associated with several diseases. Therefore, the development of analytical techniques is necessary to inform the consumer about the meat and meat products nutritional quality.

Cholesterol determination in meat and meat product was studied for decades. The first published studies were based on a previous lipid extraction followed by saponification before its quantification. Nowadays, new methods have been published that simplify the process by making direct saponification with KOH and ethanol. These methods allow a faster determination of cholesterol with less consumption of solvents. The most common chromatographic techniques for determining and quantified cholesterol in meat and meat products are gas chromatography and high-performance liquid chromatography.

This chapter gives a clear and complete vision of a rapid and precise methodology for the cholesterol analysis of meat and meat products, including all the extraction steps and the subsequent analysis by high-performance liquid chromatography with Photodiode Array detection (HPLC-DAD) using a normal phase. The conditions of extraction and analysis proposed by us are described afterwards with exactitude with the aim reproduced by other researchers.

Key words Food analysis, Cholesterol, Normal phase, HPLC-DAD

1 Introduction

Cholesterol is an essential lipid molecule that is commonly found as a component in the cell membrane [1]. One of the major functions is to participate in the biosynthesis of bile acids in the liver [2, 3]. It is an important precursor for bile acids, provitamin D, and several steroidal hormones as testosterone and estrogen [2–5]. Moreover, they also take part in the production and absorption of vitamin D [3]. Due to all the aforementioned, the intake of low cholesterol levels could have benefits for human health [3]. However, high cholesterol levels are associated with a higher risk of

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hypercholesterolemia, obesity, diabetes, brain diseases, and cardiovascular diseases [3, 6, 7].

Cholesterol is mostly present in meat, meat products and derived products from livestock as eggs, cheese, milk, butter, etc. [4]. The consumption of meat and meat products provide one third to one half of daily-recommended cholesterol intake [6]. To prevent the appearance of diseases associated with cholesterol, it is necessary accurate determination of cholesterol. The development of analytical techniques for the determination and quantification of total cholesterol in meat and meat products is important mainly to inform of their quality and safety [8].

The AOAC International adopted the first validated cholesterol determination method for foods (AOAC International 976.26, [9]). This method was based on a prior lipid extraction followed by saponification, which makes it a long and tedious method. Moreover, this extraction method increases the analysis time and the consumption of dissolvents. In order to overcome these drawbacks, the method was modified, by employing direct saponification (AOAC International 994.10, [10–12]). Nowadays, new extraction methods based on direct saponification followed by extraction with organic solvents were validated with good results [4, 8, 11, 13]. The saponification step is indispensable to separate cholesterol and other unsaponifiable materials from fatty acids and to remove triglyceride interferences [12, 14]. The most suitable direct saponification conditions are the use of an alcoholic KOH solution (between 0.33 and 0.5 M) and temperatures ranging from 55 to 75 °C during 15 to 60 min [11, 12], also known as hot saponification. Despite, cold saponification (at room temperatures) procedures during several hours (overnight) could also be used [15], to limit the cholesterol degradation during heating. It is important to highlight that in hot saponification, the addition of antioxidants (i.e., Vitamin C) and the use of anaerobic conditions (air removed with nitrogen or argon) during saponification and extraction processes contribute to preventing cholesterol oxidation [6].

For cholesterol analysis several, analytical methods have been developed, including spectroscopic and gravimetric procedures, enzymatic assays, and chromatographic methods based on gas chromatography, liquid chromatography, and mass spectrometry [1, 3]. In food, the chromatographic methods were more reliable and selective because of avoidance of the interference from other sterols [1, 4, 12, 14]. One of the most used methods in meat cholesterol analysis is based on liquid chromatography coupled to photodiode array detector [4, 8, 15].

For cholesterol analysis by HPLC, both normal phase (NP) and reversed phase (RP) can be used [13, 15]. However, due to its low polarity, the use of normal phase is preferable. In this regard, some authors proposed the cholesterol analysis by normal phase

(NP) using a polar stationary phase (silica) and an apolar mobile phase (hexane) [13]. The normal phase analysis provides better chromatograms than reversed phase methods due to the low viscosity of commonly used eluents.

Thus, the aim of this book chapter is to explain in detail a procedure for determining cholesterol in meat and meat products. The extraction consists of direct saponification (hot saponification) of the meat sample with ethanolic KOH solution, a posterior extraction with hexane, and finally the chromatographic analysis by liquid chromatography.

2 Materials

Prepare all solutions using distilled water and/or analytical grade reagents. Use HPLC-grade hexane. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing of waste materials (*see* **Note 1**).

2.1 Extraction Step

1. Saponification solution: 11% w/v potassium hydroxide (KOH) in a mixture of 55% v/v absolute ethanol (EtOH) and 45% v/v distilled water (H_2O). For 1 L of solution dissolve 129.4 g of KOH (85% purity) in 450 mL of distiller water and then add 550 mL of absolute ethanol. The saponification solution must be prepared/renewed every week.
2. BHT (25 ppm) solution in n-hexane: Dissolve 0.0125 g of butylated hydroxytoluene (BHT) in 500 mL of n-hexane.

2.2 Calibration and Cholesterol Identification

1. Cholesterol ($\geq$ 99%): For a concentration of 1 mg/mL, dissolve 0.05 g of cholesterol in 50 mL of n-hexane.
2. Standards for calibration curve: In order to obtain a seven-point calibration curve, different volumes (μL) of the previously described cholesterol standard were mixed with n-hexane (Table 1). The final concentrations (ppm) of calibration curve standards are those listed in Table 2.

Table 1
Volume (μL) of the cholesterol standard solution (1 mg/mL) and hexane for the elaboration of the different points of the calibration curve (final volume 1500 μL)

Compound	STD0	STD1	STD2	STD3	STD4	STD5	STD6
Cholesterol (1 mg/mL)	23.44	46.88	93.75	187.50	375	750	1500
Hexane (HPLC grade)	1476.56	1453.13	1406.25	1312.50	1125	750	0

Table 2
Final concentration (ppm) of cholesterol in each point of the calibration curve

Concentration (ppm)	STD0	STD1	STD2	STD3	STD4	STD5	STD6
Cholesterol	0.15625	0.3125	0.625	1.25	2.5	5	10

2.3 HPLC System

Separation and quantification of cholesterol are carried out using a liquid chromatograph model Alliance 2695 equipped with a 996 Photodiode Array Detector (Waters, Milford, MA, USA). The cholesterol separation was performed using a normal phase silica column (SunFireTM Prep Silica, 4.6 mm ID × 250 mm, 5 μm particle size, Waters, Milford, MA, USA). Empower 3TM advanced software (Waters, Milford, MA, USA) was used to control system operation and analyze results.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Cholesterol Extraction

The extraction was carried out according to the protocol described by Domínguez et al. [13] with minimal changes. Figure 1 shows a simplified and schematic way of the extraction phases.

1. Weigh 0.50 ± 0.02 g of homogenized meat sample in a screw Teflon-lined cap tube of 15 mL.
2. Add 0.25 g of L-ascorbic acid (to avoid the oxidative process) and 5 mL of saponification solution.
3. Apply nitrogen gas to eliminate the air from the reaction by displacement and then close the tube (*see* **Note 2**).
4. Shake until the ascorbic acid is completely dissolved with a vortex (approximately 30 s), then the samples are left to rest for 5 min and finally they are stirred again for another 30 s.
5. The saponification is carried out in a shaking water bath (THER-SPIN, Orto Alresa, Madrid, Spain) (200 rpm) at 85 °C for 45 min. After 20 min of the saponification process, the samples were vortexed. At the end of the saponification process, the samples were cooled at room temperature (about 10 min) (*see* **Note 3**).
6. Follow cooling, 1.5 mL of distilled water and 3 mL of 25 ppm of BHT solution in n-hexane are added and they are vortexed vigorously.
7. The samples are transferred to conical centrifuge tubes of 15 mL capacity and are centrifuged at 1500 × *g* for 3 min (*see* **Note 4**).
8. An aliquot of n-hexane (upper layer) is transferred into another tube. In order to ensure the complete dehydration of the

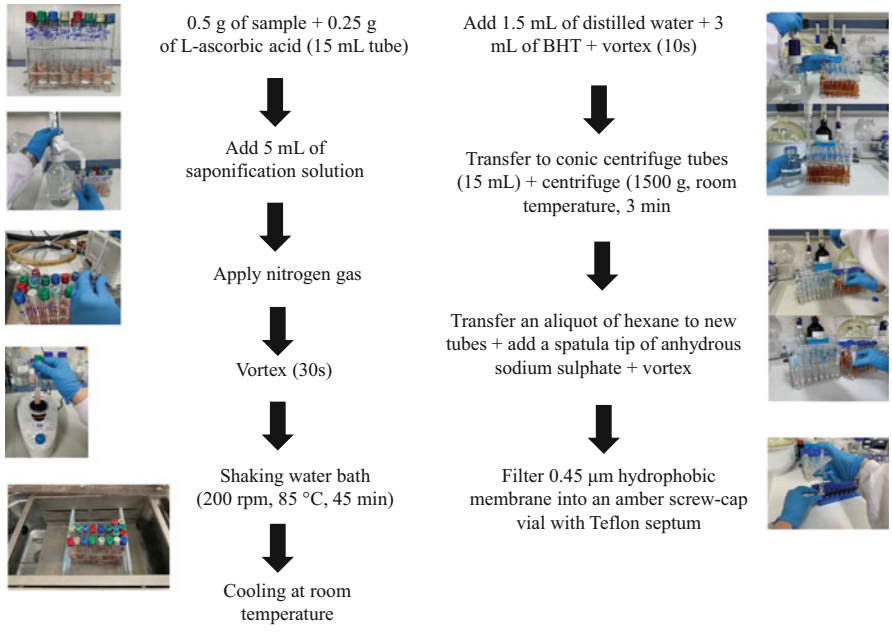

Fig. 1 Schematic and simplified representation of the steps of the cholesterol extraction phases

organic phase, a spatula tip of anhydrous sodium sulfate are added.

9. Finally, the tubes are briefly shaken (vortexed), and an aliquot of the n-hexane was filtered through a 0.45-µm hydrophobic membrane into an amber screw-cap vial with Teflon septum.

3.2 Cholesterol Identification and Quantification (HPLC-DAD)

Separation and quantification of cholesterol are carried out using a liquid chromatograph coupled with a diode array detector following the next conditions (Table 3):

1. Ten microliters of the sample (or the standard) are injected. A SunFireTM Prep Silica, 4.6 mm ID × 250 mm, 5 µm particle size, (Waters) is used for cholesterol separation. The temperature of the column oven is adjusted at 30 °C.
2. The mobile phase is adjusted at 2% 2-propanol and 98% n-hexane. The isocratic mode is used during chromatographic analysis at a flow rate of 1 mL/min.
3. The detection of cholesterol are carried out using Photodiode Array detector (DAD) at 208 nm. The total time for chromatographic analysis is 15 min.

Data acquisition, equipment control, and data analysis are carried out using the HPLC software (in our case Empower 3TM; Waters). The cholesterol in meat products is identified by comparing its retention time with the authenticated standards (Cholesterol ≥ 99%) (*see* **Notes 5** and **6**). Figure 2 shows examples of

Table 3
Summary of the chromatographic conditions used for the cholesterol analysis

Parameters	Values
Injection volume	10 µL
Flow rate	1 mL/min
Column	SunFireTM prep silica, 4.6 mm ID × 250 mm, 5 µm particle size, waters
Oven temperature	30 °C
Mobile phase	2-propanol/hexane (2:98, v/v)
Elution type	Isocratic
Detector	Photodiode Array detector
Wavelength	208 nm
Retention time	10 min
Run time	15 min

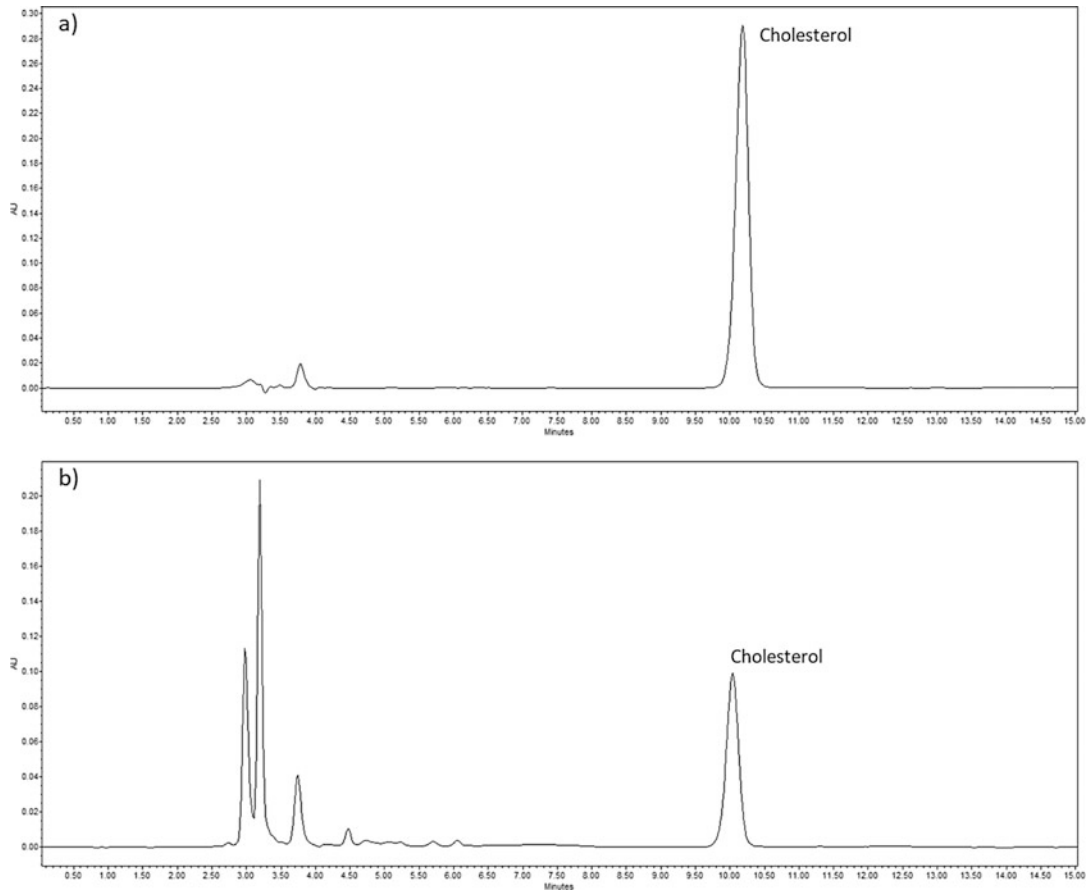

Fig. 2 Cholesterol chromatogram of a standard (**a**) and a fresh meat sample (**b**)

chromatograms of standard (a) and sample (b). The results are expressed as mg/100 g of meat.

4 Notes

1. Due to the toxic and/or corrosive nature of the solvents and reagents used in the extraction, it is necessary for the operator to take the necessary protective measures (gloves, glasses, etc.) as well as to carry out all the operations in a laboratory fume hood.
2. Apply a low pressure of nitrogen to avoid losses due to splashes.
3. The water batch should be turned on before (about 15 min) to allow the water bath to have the correct saponification temperature.
4. The emulsification of some samples may occur during the extraction process. If this happens, 0.2 mL of absolute ethanol can be added to facilitate the separation process. The ethanol is gently mixed in swirling motion, and the emulsifying mixture allowed to stand to enable the separation.
5. Standards should be injected first, for identification and to create the calibration curve. Identification/calibration standards must be prepared each time a calibration is performed. During the analysis of the samples, a blank (hexane) should be injected every 10 samples. It is recommended to inject a standard to verify retention times and the concentration each time a new sequence begins. Calibrations should be performed at least once a month, or when any maintenance is performed on the chromatograph that may affect the resolution of the equipment or the detector signal.
6. Standards prepared for calibrations can be stored frozen (−20 °C) and used to check retention times. However, these standards cannot be used for calibration, only to verify retention times. Calibration curve standards must be prepared each time a new calibration is performed.

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