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## Review Article

## Analytical methods for cholesterol quantification



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## ABSTRACT

Cholesterol is an important lipid molecule in cell membranes and lipoproteins. Cholesterol is also a precursors of steroid hormones, bile acids, and vitamin D. Abnormal levels of cholesterol or its precursors have been observed in various human diseases, such as heart diseases, stroke, type II diabetes, brain diseases and many others. Therefore, accurate quantification of cholesterol is important for individuals who are at increased risk for these diseases. Multiple analytical methods have been developed for analysis of cholesterol, including classical chemical methods, enzymatic assays, gas chromatography (GC), liquid chromatography (LC), and mass spectrometry (MS). Strategy known as ambient ionization mass spectrometry (AIMS), operating at atmospheric pressure, with only minimal sample pretreatments for real time, *in situ*, and rapid interrogation of the sample has also been employed for quantification of cholesterol. In this review, we summarize the most prevalent methods for cholesterol quantification in biological samples and foods. Nevertheless, we highlight several new technologies, such as AIMS, used as alternative methods to measure cholesterol that are potentially next-generation platforms. Representative examples of molecular imaging of cholesterol in tissue sections are also included in this review article.

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## 1. Introduction

Cholesterol is a sterol lipid produced by animal cells. It is a relatively small biological molecule that plays important roles in human body. Most of cholesterol is produced in liver, adrenal glands, intestines, and in gonads, whereas 20–25% of cholesterol comes from the diet of animal origin [1].

Cholesterol is an essential component of all cell membranes - it helps to maintain structural integrity and fluidity of cell membranes, allowing animal cells to change shapes easily without cell walls [2]. One of the major functions of cholesterol is to participate in the biosynthesis of bile acids in the liver [3]. Bile can break down dietary fats into smaller droplets and helps the subsequent digestion. Cholesterol also participates in the absorption and the production of vitamin D [4].

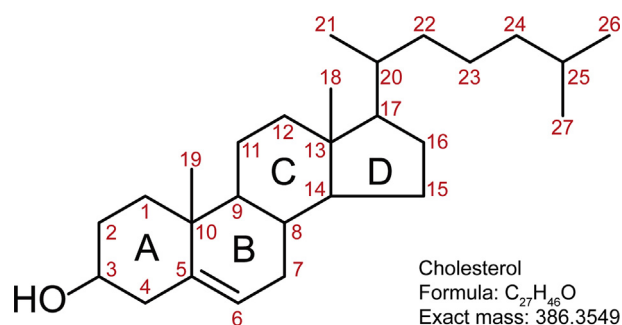
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**Fig. 1 – Structure of cholesterol with the numbering of the carbon atoms.**

Moreover, cholesterol is a precursor of steroids, such as testosterone and estrogen [5]. Besides, cholesterol is also a key component in lipoproteins, which are used to transport hydrophobic molecules (such as fats) in hydrophilic media, like blood. Lipoproteins, classified as very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), intermediate-density lipoprotein (IDL), and high-density lipoprotein (HDL), consist of esterified and free cholesterol, proteins, phospholipids, and triglycerides. Cholesteryl ester transfer protein (CETP) facilitates transfer of cholesterol esters and triglycerides among different classes of lipoproteins [1].

A cholesterol molecule contains three major parts: 1) tetracyclic carbon ring (A–D) as the core of steroids, 2) polar hydroxyl group attached to ring A, and 3) short non-polar carbon chain attached to ring D (Fig. 1) [6,7]. All four rings of the sterol group are in a trans conformation, making cholesterol a planner molecule. The double bond between  $C_5$  and  $C_6$  helps to keep the rigidity of cholesterol.

Cholesterol is required for viability and cell proliferation. More than 90% of cellular cholesterol is located at the plasma membrane [8]. The cholesterol molecule is inserted into lipid bilayers, where it aligns itself parallel to the glycerophospholipids and sphingophospholipids. The hydroxyl group is very close to the polar lipid head but is insufficient to shield the hydrophobic ring from water, thus, cholesterol tails are very closely associated with saturated phospholipid and sphingolipids where the enzymes, substrates, and products involved tends to be extremely hydrophobic [7]. Thus, cholesterol has vital structural roles in membranes and in lipid metabolism with the extraordinary diversity of biological roles [9–11].

In this article, we discuss the importance of cholesterol measurement in clinical and food sciences, introducing the most prevalent methods used for cholesterol quantification in the related fields. In the end, we highlight several new analytical platforms that are potentially the next-generation techniques in clinical laboratories.

## 2. Why measuring cholesterol?

### 2.1. Cholesterol in clinical sciences

Cholesterol is an essential building block for cell membranes and also important precursor for production of hormones [1].

Up to certain levels, cholesterol is beneficial for human body. Meanwhile, higher levels of cholesterol are associated with many diseases. According to the National Heart Lung and Blood Institute (NHLBI, USA), the higher the level of LDL cholesterol in our system, the greater our chances of getting heart diseases. On the other hand, people with higher level of HDL cholesterol have lower chances of getting heart diseases [12,13]. LDL carries cholesterol around the body to the parts where it is demanded. However, if there is too much of cholesterol in the bloodstream, it tends to cling to the walls of the arteries and may eventually lead to the clogging. Excessive cholesterol can deposit in the arteries and promote formation of plaques, leading to atherosclerosis [14]. Therefore, LDL is referred to as “bad cholesterol”. In the contrary, HDL is called as “good cholesterol” due to its capability to gobble up cholesterol and to take it back to the liver [15]. To determine lipoprotein level and obtain complete lipoprotein profile, blood sample is withdrawn after 12 h fasting, while to determine total level of cholesterol - fasting is not required [13]. The concentration of total cholesterol, occurring in the free form and as cholesterol esters (esterified to long-chain fatty acids), in human serum is highly variable among individuals. The American Heart Association (AHA, USA) recommends all adults at age between 40 and 75, without cardiovascular diseases and with LDL-C between 70 mg/dL (1.81 mmol/L) and 189 mg/dL (4.90 mmol/L), to take tests for cholesterol levels every 4–6 years [15,16].

The association between elevated total cholesterol and the risk of cardiovascular disease – one of the leading causes of death worldwide – is well documented [17,18]. In addition, abnormal levels of cholesterol or its precursors also have been observed in various human diseases, such as Smith-Lemli-Opitz syndrome (SLOT) [19], non-insulin-dependent diabetes mellitus [20], brain diseases [21], and cancers [22]. Therefore, regular monitoring of cholesterol level is important for individuals who are at increased risk of these diseases. As cholesterol is an early-warning indicator of various diseases, screening tests for cholesterol are mandatory in adult healthcare in clinical laboratories, and its levels are determined in routine blood tests. According to the National Cholesterol Education Program (NCEP, USA), optimal LDL and total cholesterol concentrations are < 100 mg/dL and < 200 mg/dL, respectively. HDL concentration < 40 mg/dL is considered as low, while  $\geq 60$  mg/dL is considered as high [13].

### 2.2. Cholesterol in food sciences

Public interests in dietary cholesterol had increased considerably due to the association of plasma cholesterol levels with the risk of heart diseases. As around 20–25% of cholesterol in our body comes from the food of animal origin, such as egg, meat, milk products, etc., it is important to know the cholesterol concentration in our dietary intake [23]. Food nutritional information need to be provided by laboratories to food producers for precise food labeling, as a nutrition food label has to inform customers about healthy eating and appropriate food choices to reduce nutrient-related diseases [24]. Thus, evaluation of the exact cholesterol content in foods is crucial and drives the technological development for cholesterol quantification [25,26].

**Table 1 – Summary of the methods described in the article.**

Method	Characteristics	Ref.
Modified Abell-Kendall method	This is a standard reference method for total blood cholesterol measurement. It is a multi-step classical chemical method, which involves saponification of cholesterol esters by hydroxide, extraction with hexane, and color development with acetic anhydride–sulfuric acid.	[28]
Fluorometric-enzymatic assay	This assay is based on an enzyme-coupled reaction for determining free cholesterol and cholesterol esters. Esterified cholesterol is converted to cholesterol by cholesterol esterase. The resulting cholesterol is then acted upon by cholesterol oxidase to produce cholest-4-en-3-one and hydrogen peroxide and the corresponding ketone product. Hydrogen peroxide is then detected using sensitive and stable fluorescence probe.	[29] [30] [31]
GC/GC-MS: gas chromatography – mass spectrometry	This method is used for routine cholesterol determination. It is used to separate cholesterol from other interfering species. It can be used for determination of free, esterified and total cholesterol levels (based on sample pretreatment). More accurate than classical chemical and enzymatic methods. Extensive sample preparation (including derivatization) prior analysis is required. Labor and resource intensive.	[32] [33] [34]
GC-ID-MS	This is a standard reference method for total blood cholesterol measurement. Isotopically labeled cholesterol is added to a sample in an amount approximately equal to the actual amount of analyte present in a sample. Additionally, two calibration mixtures of a known cholesterol and labeled cholesterol concentrations are prepared. Quantification is based on calculating peak area ratio corresponding to unlabeled and labeled cholesterol for unknown sample and comparing it with the ratios calculated for calibration mixtures.	[27]
LC/LC-MS: liquid chromatography – mass spectrometry	This method is used for routine cholesterol determination. Similarly to GC, it is used to separate cholesterol from other interfering species. It can be used for determination of free, esterified and total cholesterol level (based on sample pretreatment). More accurate than classical chemical and enzymatic methods. Extensive sample preparation prior analysis is required. Labor and resource intensive.	[35] [36]
ESI-MS/MS: electrospray ionization tandem mass spectrometry	This method requires derivatization to increase ionization efficiency. Free cholesterol may be converted to cholesteryl acetate using acetyl chloride. Sample is directly injected to MS. Fragment at $m/z$ 369 is used for quantification (SRM mode and precursor ion scan).	[37]
MALDI-MS: matrix-assisted laser desorption ionization-time of flight	This method requires use of matrix. Signal at $m/z$ 369 corresponding to dehydrated molecule $[M-H_2O+H]^+$ can be observed. Lipoprotein lipid signals in 369–910 $m/z$ range can be detected in human serum.	[38]
MALDI-IM-MS: Matrix-assisted laser desorption/ionization-ion-mobility mass spectrometry	This method takes advantage of ion-mobility to separate chemical noise from the analytes of interest. Coating samples with metal increases the conductivity of insulating substrates, such as tissues or cells. Using sputtered silver nanoparticles instead of matrix provides lower limit of detection based on signals of silver coordinated molecules. Profiling of cholesterol and 7-dehydrocholesterol in SLOS human fibroblast can be done.	[39]
DESI-MS: Desorption electrospray ionization mass spectrometry	This method often requires derivatization to increase ionization efficiency. Derivatizing reagent can be incorporated into DESI spray. Betaine aldehyde reacts with cholesterol forming charge-labeled cholesterol.	[40]
DART: Direct analysis in real time mass spectrometry	This method is suitable for the analysis of non-polar compounds. Signal at $m/z$ 369 corresponding to dehydrated molecule $[M-H_2O+H]^+$ can be observed. This method can be used for food and biological samples analysis. Endogenous cholesterol from serum spotted onto the chromatography paper can be desorbed and rapidly ionized. Fast quantitative screening can be achieved.	[26] [41]
MALDI-MSI: Matrix-assisted laser desorption/ionization mass spectrometry imaging	This method requires covering tissue section with a thin layer of matrix. It can be used to determine the distribution of endogenous cholesterol sulfate as well as exogenous compounds in human skin tissue sections. Spatial resolution is around 30 $\mu\text{m}$ .	[42]
DESI-MSI: Desorption electrospray ionization mass spectrometry imaging	This method allows interrogation of the sample in its native environment. No matrix is required. Reactive DESI (with a betaine aldehyde as a reagent) can be used to image cholesterol in rat brain tissue. Spatial resolution is around 200 $\mu\text{m}$ . DESI can be used to image cholesterol sulfate that is a differentiating compound between healthy and prostate cancer tissue.	[22] [40]

### 3. Methods for cholesterol measurements

Multiple methods have been developed for cholesterol levels determination. Those methods can be divided into three major categories: 1) classical chemical methods based on the Abell-Kendall protocol, 2) fluorometric and colorimetric enzymatic assays that are commonly employed for assay kits and automated plate readers, and 3) analytical instrumental approaches such as gas and liquid chromatography or mass spectrometry. Each of those methods has its distinctive advantages. 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 (LOD) are usually low. Chromatographic and mass spectrometric methods are the most accurate and sensitive ones, however they require costly equipment and extensive sample pretreatment. National Institute of Standards and Technology (NIST, USA) has approved an isotope-dilution mass spectrometry (ID-MS) method [27] and modified Abell-Kendall protocol [28] as two reference measurement procedures (RMPs) for blood cholesterol quantification. Based on those methods, various analytical protocols have thus been developed. Recently, ambient ionization mass spectrometry techniques (AIMS) have been proposed as alternative approaches for high-throughput screening as they do not require tedious sample pretreatments. We summarize several noteworthy methods for cholesterol analysis in Table 1 and discuss them in more details in the following paragraphs. In this review article, we focus more on description of analytical instrumental approaches and we highlight several new technologies, such as AIMS, used as alternative methods to measure cholesterol that are potentially next-generation platforms. Representative examples of molecular imaging of cholesterol in tissue sections are also included here.

In addition to the selection of the appropriate analytical technique, a proper sample preparation is also crucial in cholesterol analysis. Cholesterol esters constitute about two-thirds of the cholesterol in the blood and can be hydrolyzed chemically by a hydroxide (saponification) or enzymatically by cholesterol esterase to free cholesterol for total cholesterol quantification. For serum cholesterol quantification, at least 0.5 mL of blood specimen is drawn and around 15  $\mu$ L of serum is prepared for analysis. For plant- and animal-derived foods cholesterol determination, a sterol fraction is isolated from lipid extract by thin-layer or column chromatography, following hydrolysis [43,44]. All these methods commonly share the same procedure for sample preparation that includes saponification extraction step of total lipids and a multistage solvent extraction followed by purification and concentration.

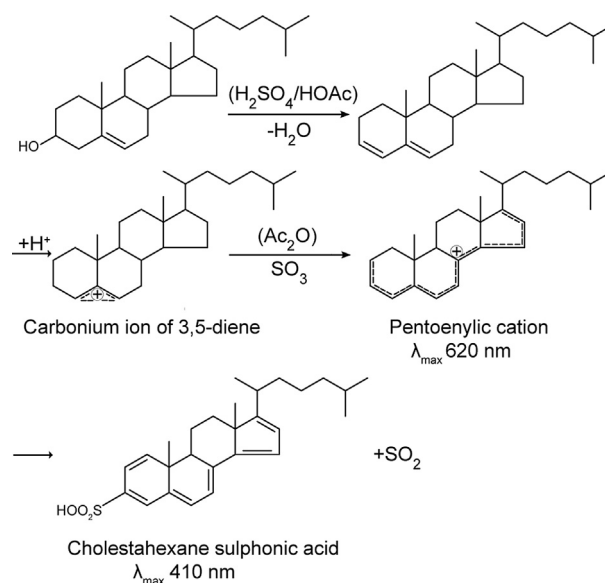
Quantitation of cholesterol in foods is often achieved by gas chromatography with flame ionization (GC-FID) or mass spectrometry (GC-MS) detection, spectrophotometry, and high performance liquid chromatography (HPLC). For milk and related products, cholesterol is initially isolated from the crude materials by solid-phase extraction (SPE). Such protocols have been established by The Association of Official Analytical Chemists (AOAC, USA) and International Dairy

Federation (IDF) [45–47]. Recently, a rapid and reliable method for cholesterol determination in egg-containing foods by direct analysis in real time mass spectrometry (DART-MS) was reported [26].

High throughput liquid chromatography tandem mass spectrometry (LC-MS/MS) has become a gold standard method for measurement of steroid substances in biological fluids and foods. Gas chromatography mass spectrometry (GC-MS) is also widely used to quantify cholesterol. Details of these methods are elaborated in the following paragraphs.

#### 3.1. Modified Abell-Kendall method

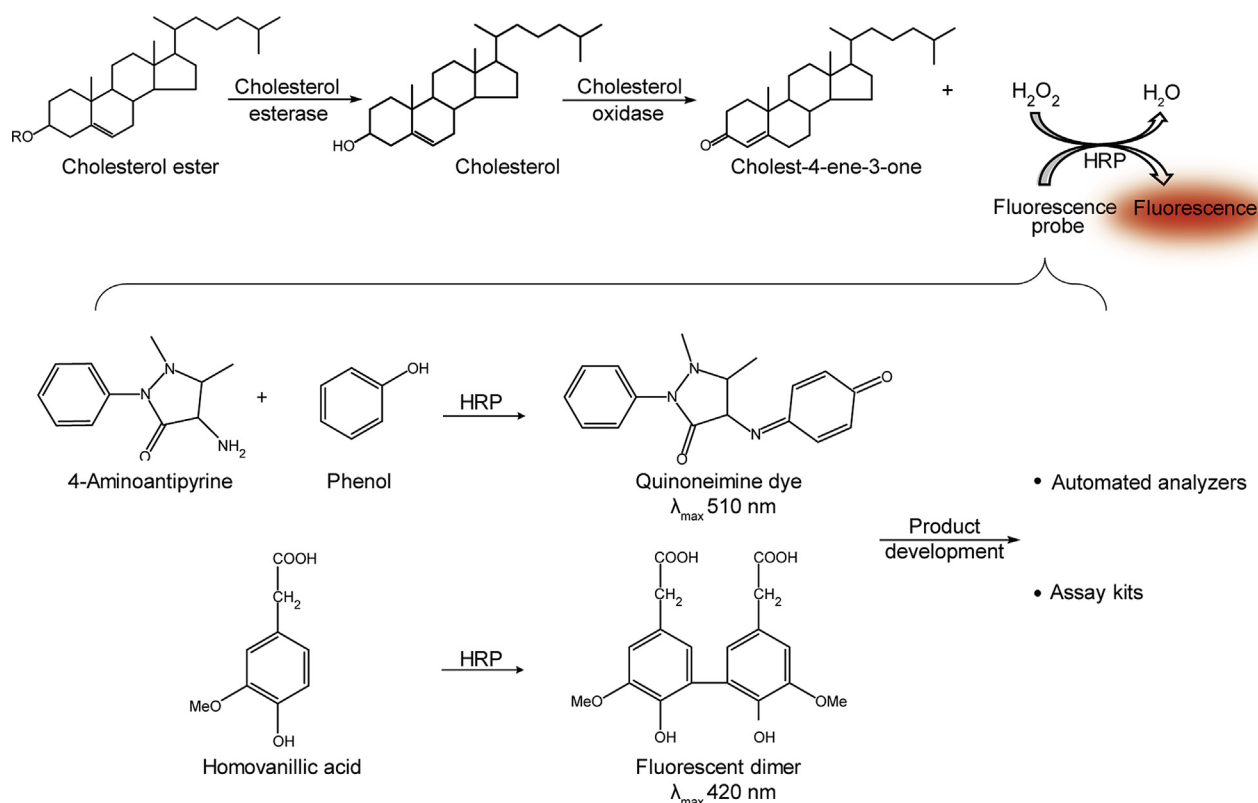
The modified Abell-Kendall method is a standard reference method for determination of total cholesterol for clinical use approved by NIST. It is a multi-step classical chemical method involving Liebermann-Burchard (L-B) reaction for color development [28]. It includes saponification of cholesterol ester with alcoholic potassium hydroxide, extraction of hydrolyzed cholesterol with hexane (instead of petroleum ether as in original Abell-Kendall method) followed by evaporation of the solvent, and finally color development with acetic anhydride and concentrated sulfuric acid. The scheme of L-B reaction is shown in Fig. 2. Appearance of the color is due to the reaction of hydroxyl group of cholesterol. Ultra-violet (UV) spectrophotometry ( $\lambda = 410$  nm) is commonly used for measurement in order to quantify cholesterol. Although, modified Abell-Kendall reaction is considered as a standard reference method, its use is not accepted for routine tests nowadays since highly corrosive reagents are used for L-B reaction.



**Fig. 2** – The scheme of the Liebermann-Burchard reaction for color development in modified Abell-Kendall method [28].

#### 3.2. Enzymatic assays

Those types of assays are based on the enzyme-coupled reaction to determine total cholesterol levels. The first enzymatic assay to measure cholesterol in serum was introduced



**Fig. 3** – The scheme of the enzyme-coupled reaction of fluorometric enzymatic assay.

in 1974 [29]. Since then, enzymatic assays started being commonly used and they are widely employed for assay kits and automated analyzers nowadays [48]. At first, esterified cholesterol is hydrolyzed to free cholesterol by cholesterol esterase (see Fig. 3) [29–31]. The free cholesterol is subsequently oxidized to cholest-4-en-3-one by cholesterol oxidase. Hydrogen peroxide is produced in this reaction as a side product and can be easily detected using high sensitive colorimetric or fluorometric probes. Multiple fluorometric probes have been developed and therefore enzymatic assays have become a common strategy. For example, 4-aminoantipyrine (with addition of phenol known as Trinder reagent) [29], homovanillic acid [49], and 10-acetyl-3,7-dihydroxyphenoxazine (known as Amplex Red) [30] can be catalyzed by horse radish peroxidase (HRP) to form the fluorescent product in a presence of hydrogen peroxide (see Fig. 3). For routine analysis, many laboratories use commercial cholesterol quantitation assay kits, handheld point-of-care testing (POCT) devices and automated analyzers based on enzymatic assays [31,48]. It should be mentioned, that enzymatic assays may not be strictly selective for cholesterol determination. In fact, cholesterol oxidase may also react with other sterols [50]. Moreover, some chemicals present in a sample (such as ascorbic acid and bilirubin) may consume hydrogen peroxide and some bias during indirect cholesterol quantification may occur.

### 3.3. Gas and liquid chromatography

Chromatographic techniques such as gas (GC, where gas is a mobile phase) and liquid (LC, where liquid is a mobile phase)

chromatography are commonly used for cholesterol quantification [32,51]. Those methods are widely accepted to be more reliable, sensitive and accurate than the other methods. Additional selectivity is achieved due to the fact that cholesterol is separated from interfering species (such as sterols) on chromatographic columns. Moreover, low volumes of samples (tens of  $\mu\text{L}$ ) are required for analysis. Meanwhile, as mentioned before, the performance of chromatographic techniques are more dependent on sample preparation, such as saponification and analyte extraction. Analysis of cholesterol and cholesterol esters is the most often accomplished by GC. In 1964, GC coupled with hydrogen flame ionization detector was employed to analyze cholesterol in serum [32]. In 1979, reverse phase high-performance liquid chromatography (HPLC) with an UV detection at  $\lambda = 210$  nm was used for determination of free, esterified and total cholesterol in serum [35]. Linear range from 0.09 to 0.31 g/L for quantification of cholesterol was achieved.

Cholesterol is readily ionized by electron ionization (EI), but other MS ionization techniques including electrospray ionization (ESI) are less effective due to low proton affinity and low acidity of cholesterol [40]. Methods based on mass spectrometry usually use isotopically labeled cholesterol ( $^2\text{D}$  labeled: cholesterol- $\text{D}_6$ , cholesterol- $\text{D}_7$  or  $^{13}\text{C}$  labeled) as internal standards to compensate for signal variability. Using internal standard, more accurate quantification by MS is achieved. GC coupled with MS detection was used for analysis of cholesterol, in which total free cholesterol was extracted from serum with chloroform and derivatized with bis(trimethylsilyl)tri-fluoroacetamide (BSTFA) [34]. Commonly used step of saponification was omitted. Linear range from 0.1 to 15 mmol/L for

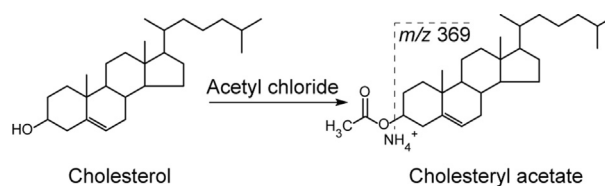
cholesterol quantification was achieved. The limit of detection (LOD) was 0.04 mmol/L and intra-day and inter-day precision and recovery were 92.5%–98.5% ( $n = 6$ ). It was found that total cholesterol levels in rats serum positively correlated with high cholesterol diet.

Isotope-dilution mass spectrometry (ID-MS) is a reference measurement procedure for cholesterol quantification approved by NIST. In 1980, the first GC-ID-MS method was presented [27]. In this report, cholesterol- $D_7$  was added to the serum sample in a specific amount, so that unlabeled to labeled cholesterol peak area ratio was approximately equal to 1. Then, cholesterol esters were hydrolyzed and resulting free cholesterol, along with cholesterol- $D_7$ , were extracted and derivatized for GC-MS analysis. Additionally, two calibration mixtures of a known cholesterol and cholesterol- $D_7$  concentrations, with unlabeled to labeled cholesterol peak area ratio from 0.8 to 1.2, were prepared following the same protocol. Quantification was based on calculating peak area ratio corresponding to unlabeled and labeled cholesterol for unknown sample and comparing it with the ratios calculated for calibration mixtures (so called bracketing protocol). Later on, various modifications of this method were developed and presented. For example, liquid chromatography–isotope dilution mass spectrometry (LC-ID-MS) employing cholesterol- $^{13}C_3$  was reported [36]. Initially, alkaline hydrolysis and cholesterol extraction were performed. No derivatization was used this time, making it a convenient and popular method. High accuracy and precision were achieved and good stability of the method was reported.

### 3.4. Electrospray ionization tandem mass spectrometry

Electrospray ionization (ESI) is not an effective ionization technique for neutral free sterol molecules such as cholesterol. Molecular ions  $[M+H]^+$  of sterol molecules fragment easily in the ion source, therefore it is difficult to detect them. In contrary, cholesterol ester species tend to form more stable ammonium adduct ions  $[M+NH_4]^+$  that can be successfully detected. Derivatization of free cholesterol is required to improve ionization efficiency in ESI-MS. For example, cholesterol can be converted to cholesteryl acetate in a reaction with acetyl chloride (see Fig. 4). Such a procedure was used to simultaneously analyze free cholesterol and cholesterol esters in plasma samples and cell homogenates by ESI-MS/MS [37]. In this report, free cholesterol was converted to cholesteryl acetate. Upon collision induced dissociation, derivatized cholesterol as well as cholesterol esters ( $[M+NH_4]^+$  ions), formed the same fragment ion at  $m/z$  369 and thus it was used for further quantification. Cholesterol- $D_7$  served as internal standard. Selected reaction monitoring (SRM) in a combination with precursor ion scan mode was used.

Alternatively, silver cationization approach can be implemented for direct cholesterol ionization due to high affinity of  $Ag^+$  ions to  $\pi$  orbitals. Electrolytically generated metal cations (such as  $Ag^+$ ) can be injected from the primary electrospray towards an open-tube secondary capillary containing the sample. Such a method defined as relay ESI (rESI) was successfully applied to form stable  $[M+Ag]^+$  cholesterol ions at  $m/z$  493 and to perform MS/MS analysis for 100 ppb of the analyte [52].



**Fig. 4** – Scheme of cholesterol derivatization with acetyl chloride to form cholesteryl acetate. Fragment at  $m/z$  369 is used for quantitative purposes in ESI-MS/MS analysis [37].

### 3.5. Matrix-assisted laser desorption/ionization mass spectrometry

Matrix-assisted laser desorption/ionization (MALDI) is not an effective ionization technique for neutral free sterol molecules. Nevertheless, there are increasing evidences showing that MALDI-MS can be used in cholesterol measurements. In the study by Hidaka et al., MALDI-TOF (time-of-flight) MS was employed for the analysis of cholesterol in human serum lipoproteins [38]. Lipids were isolated and extracted from serum and further mixed with the organic matrix (2,5-dihydroxybenzoic acid; 2,5-DHB) dissolved in chloroform-methanol (Folch's solution). Lipoprotein lipid signals were detected in a range of 369–910  $m/z$ , while signals from 2,5-DHB matrix were observed in a range of 154–551  $m/z$ . Signal from dehydrated cholesterol was detected at  $m/z$  369  $[M-H_2O+H]^+$ . Quantitative measurement was achieved by using 4-cholesten-3-one as an internal standard to compensate the instrument variation.

Silver cationization was implemented to enhance ionization of cholesterol by infrared matrix-assisted laser desorption/ionization (IR-MALDESI). Trace amounts of silver nitrate ( $AgNO_3$ ) were doped into the ESI solvent. Authors observed that adduct ion intensity was proportional to the concentration of the dopant in the solvent up to specific value (100  $\mu$ mol/L). Selectivity of this approach was reasonable and cholesterol detection was possible in complex sample such as human serum [53].

In addition, sputtered silver matrix-assisted laser desorption/ionization-ion mobility-mass spectrometry (MALDI-IM-MS) strategy for profiling cholesterol and 7-dehydrocholesterol (7-DHC) in human fibroblast cells was reported [39]. In the study by Xu et al., sputtered silver nanoparticles provided a LOD as low as 0.5 pmol/ $mm^2$ . Ion-mobility was implemented to separate chemical noise from the analytes of interest to improve LOD. The 7-DHC/cholesterol ratios were compared between Smith-Lemli-Opitz syndrome (SLOS) and control human fibroblasts, revealing a higher cholesterol level in SLOS cells. This result indicates the possibility to investigate cholesterol biosynthesis disorders using mass spectrometry-based methods [39].

## 4. Ambient ionization mass spectrometry

In the past decade, a strategy known as ambient ionization mass spectrometry (AIMS) was developed for chemical analysis on biological systems [54]. Ambient ionization techniques

operate at atmospheric pressure and they require only minimal sample pretreatment for real time, *in situ*, and rapid mass spectrometric analysis. Desorption electrospray ionization (DESI) [55] and direct analysis in real time (DART) [56] are two rapid AIMS-based technologies that can be directly used for quantitative cholesterol measurements and are more effective than direct ESI approaches for cholesterol ionization.

#### 4.1. Desorption electrospray ionization mass spectrometry

DESI is an electrospray-based ion source developed in 2004 by Takáts et al. [55]. Streams of electrically charged droplets are directed toward the sample surfaces (see Fig. 5a). Charged micro-droplets desorb molecules from the surface, where gas-phase ions are generated and subsequently transferred toward the mass spectrometer. Although DESI MS can be used for analysis of non-polar compounds, yet ionization efficiency is not satisfactory. Therefore, derivatization is usually required for cholesterol measurement.

In this regard, application of reactive DESI is utilized to improve the sensitivity and selectivity. For example,

betaine aldehyde was incorporated into the DESI spray solvent to react with the hydroxyl group of cholesterol through nucleophilic addition and form a hemiacetal salt (see Fig. 5b) [40]. Using this approach – called charge labeling, free cholesterol was quantified in serum samples with LOD reduced from over 200 ng for conventional DESI to 1 ng for reactive DESI.

#### 4.2. Direct analysis in real time mass spectrometry

DART is a plasma-based ion source developed in 2005 by Cody et al. [56]. Metastable species that are generated by an excited helium or argon gas stream react with analytes transforming them into gaseous ions. DART is mainly used for ionization of relatively non-polar compounds with molecular weight lower than 1500 Da. Due to the fact that DART ion source is available commercially, it has become the most commonly adopted AIMS technique in the world. For cholesterol, signal at  $m/z$  369 corresponding to dehydrated molecule ions  $[M-H_2O+H]^+$  can be observed.

A rapid method, based on DART MS, was developed for quantitation of cholesterol in egg-containing food products

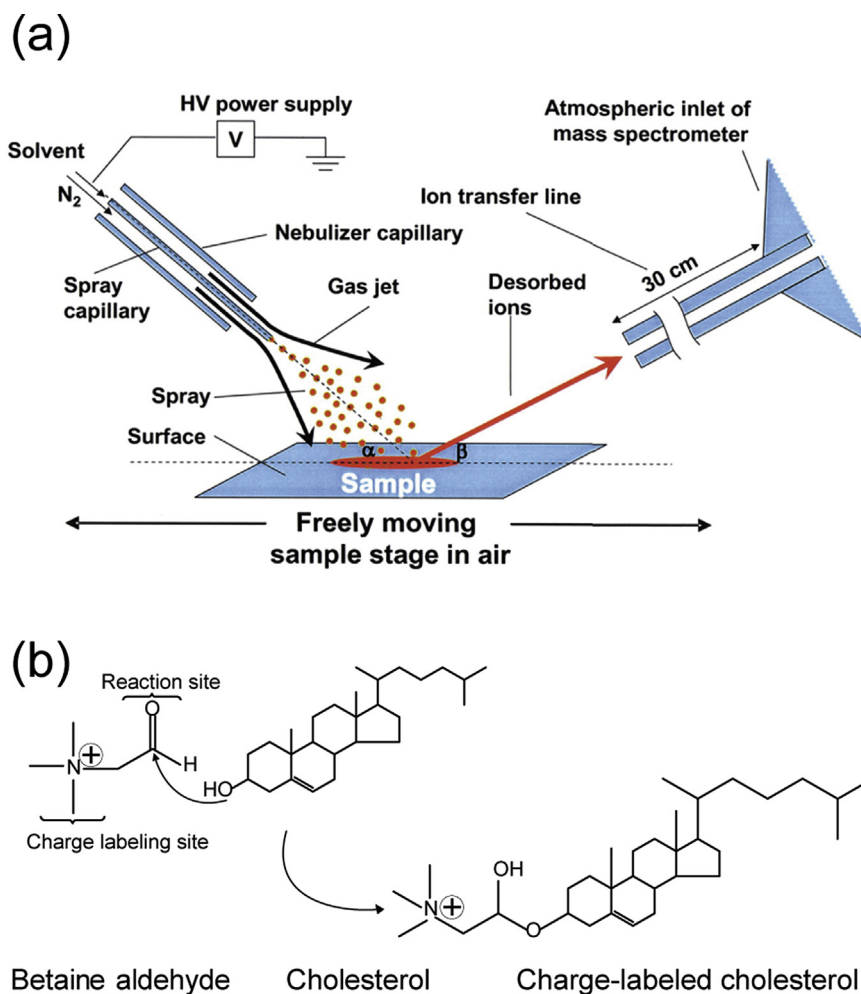
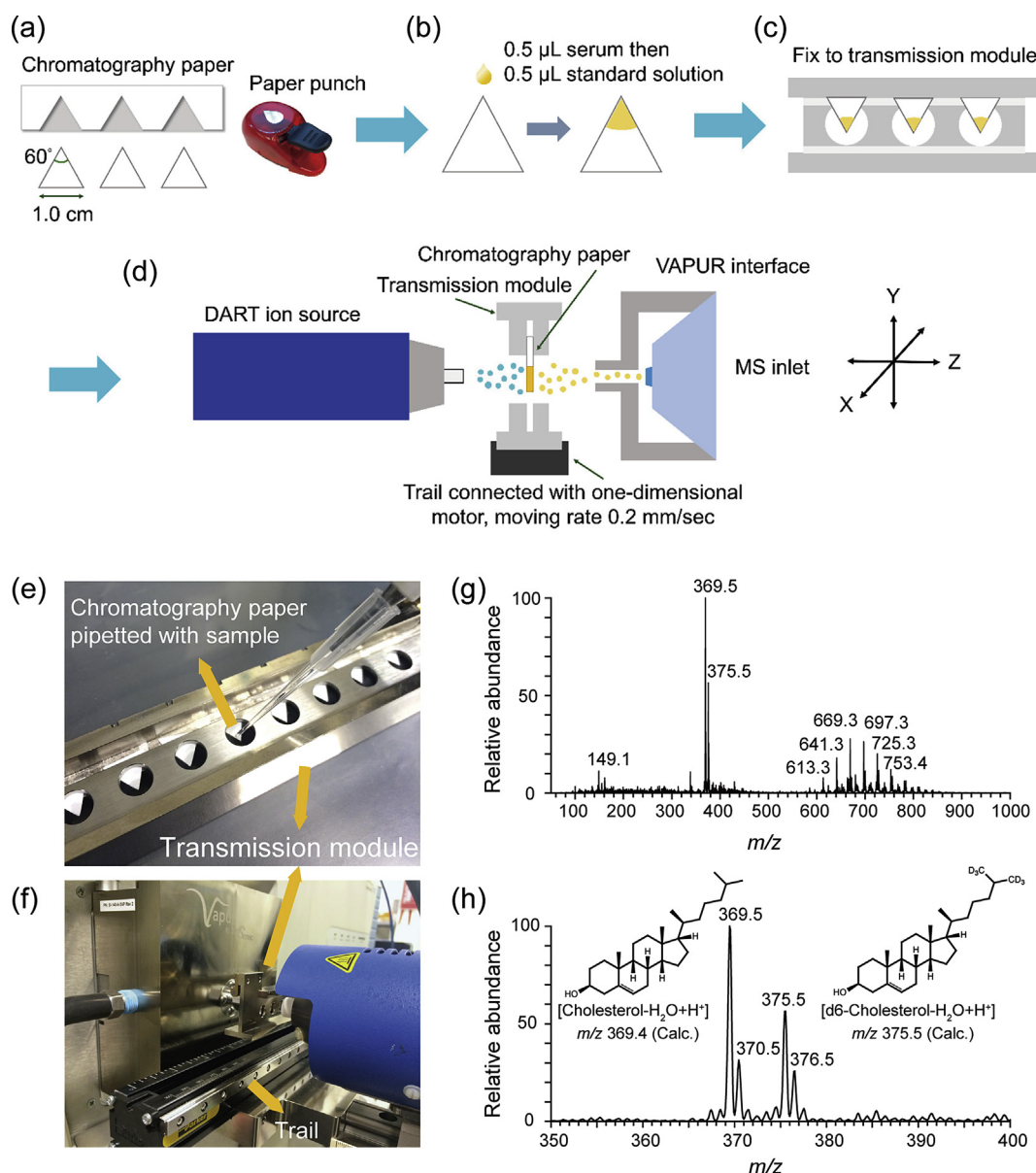


Fig. 5 – (a) Desorption electrospray ionization mass spectrometry instrumental setup. Reprinted with permission from Ref. [55]. (b) Charge labeling reaction using betaine aldehyde for reactive DESI [40].

such as dried eggs or egg pasta [26]. Following simple solvent extraction, samples were introduced to the ionization region using “Dip-it” tips. The sampling tips were immersed into vials containing sample solutions and transferred to the optimized position on the holder. The mounted tips were sequentially exposed to helium gas stream for ionization of analytes. Cholesterol- $D_6$  was used as internal standard for quantification. Although the LOD for cholesterol in real samples (0.03 mg/g) was not as low as for conventional GC-FID analysis (0.002 mg/g), this new methodology based on DART is much simpler and faster. Capabilities for quantitative analysis of the method are suitable for preliminary screening.

### 4.3. Direct analysis in real time mass spectrometry of serum cholesterol

A method for rapid and cost-effective screening of endogenous free cholesterol in human serum samples was presented using DART ion source (see Fig. 6) by Hsieh et al. [41]. Small volume of the serum (0.5  $\mu$ L) was loaded onto a tiny piece of chromatography paper followed by loading the same volume of internal standard (Fig. 6a,b). Subsequently, the paper substrates were mounted on the transmission module that was moved on the trail by a motor towards DART ionization region for mass spectrometric interrogation (Fig. 6c–f). Such methodology was abbreviated as pDART-MS. Cholesterol from



**Fig. 6 – Workflow of pDART-MS for serum cholesterol quantification: (a) cutting chromatography papers, (b) loading sample and internal standard onto paper, (c) fixing paper substrate with loaded sample on the transmission module, (d) scanning across the samples. Experimental setup of pDART-MS: (e) and (f). Typical mass spectra of serum samples: (g) positive ion mode spectrum of a mixture of serum sample and cholesterol- $D_6$ , (h) magnification of the mass spectrum. Adapted with permission from Ref. [41].**



serum was efficiently desorbed from the paper substrate and rapidly reacted with the plasma-induced metastable species. Signal at  $m/z$  369.5, corresponding to dehydrated cholesterol, was the dominant signal in the mass range of 50–1000  $m/z$  (Fig. 6g–h). The optimized method enabled the detection of cholesterol at the level of around 20  $\mu\text{g/mL}$  in serum. The concentration of cholesterol in pooled serum samples was estimated to be 250  $\mu\text{g/mL}$  and it was in very close agreement with the results obtained by LC-MS and fluorometric-enzymatic assays. Using this technology, concentration of cholesterol in each serum samples could be assessed within only 1 min. Therefore, large number of samples can be analyzed in short period of time. Presented methodology was applied to test blood samples collected from 21 recreational runners participating in the ultramarathon race. As a result, pDART-MS demonstrates a potential complementary tool for cholesterol tests in clinical laboratories and could possibly be translated to the use for POCT.

### 5. Mass spectrometry imaging of cholesterol in biological tissues

Visualizing the distribution of molecules in biological tissues enables the understanding of physiological processes and

disease pathology [57,58]. The first generation of molecular imaging of tissue sections mainly targets for proteins and is largely relied on the use of immunohistochemistry [59]. However, using this traditional method it was difficult to assess spatial distribution of other compounds such as lipids. However, the second generation of molecular imaging, mass spectrometry imaging (MSI) in specific, aims for determining *in situ* spatial localization of a wide variety of molecules including metabolites, lipids, and polypeptides. MALDI-MSI was formally introduced in 1997 by Caprioli group and has soon become an effective and reliable technique [60]. MALDI-MSI uses organic matrix to cover tissue sections so as to facilitate ablation and ionization of biomolecules. However, matrix causes interferences in low mass range and therefore imaging of low molecular weight species is troublesome and therefore MALDI-MSI was mostly employed to localize peptides and proteins [60]. MALDI-MSI was also used to determine localization of endogenous cholesterol sulfate, which is formed from cholesterol by steroid sulfotransferases, in human skin tissue sections [42]. For this purpose, corona discharge treatment was used to modify the indium–tin oxide-coated glass slide surface to improve adhesion of the tissue. Localization of cholesterol sulfate in skin tissue was demonstrated with spatial resolution of 30  $\mu\text{m}$  (see Fig. 7). Cholesterol sulfate was found to be present predominantly in

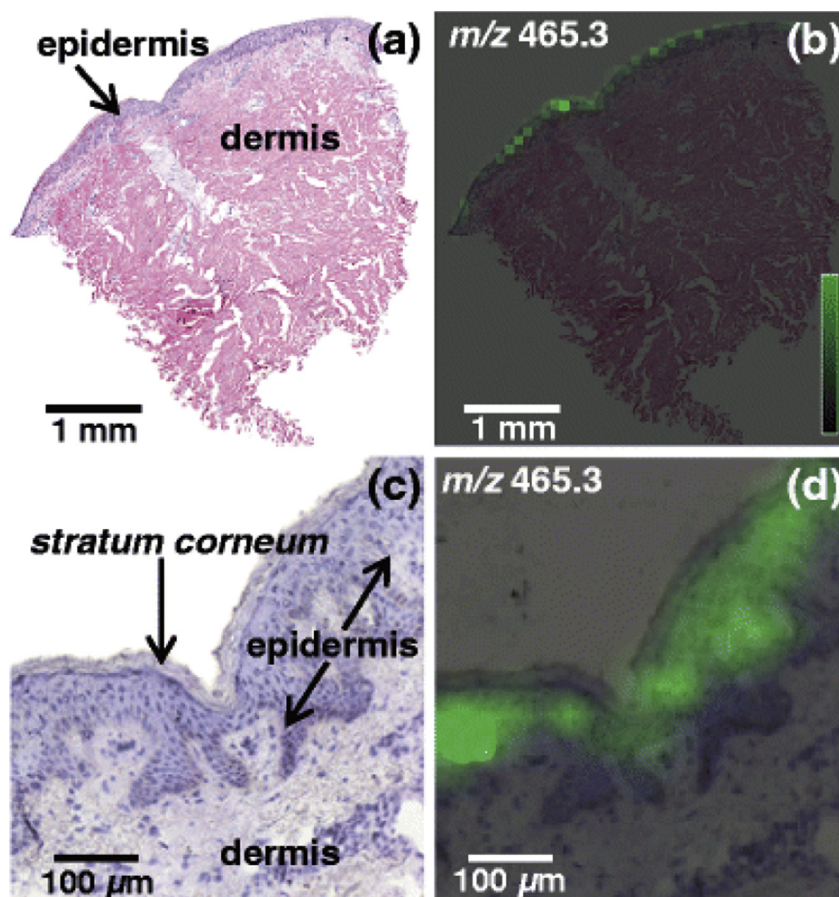
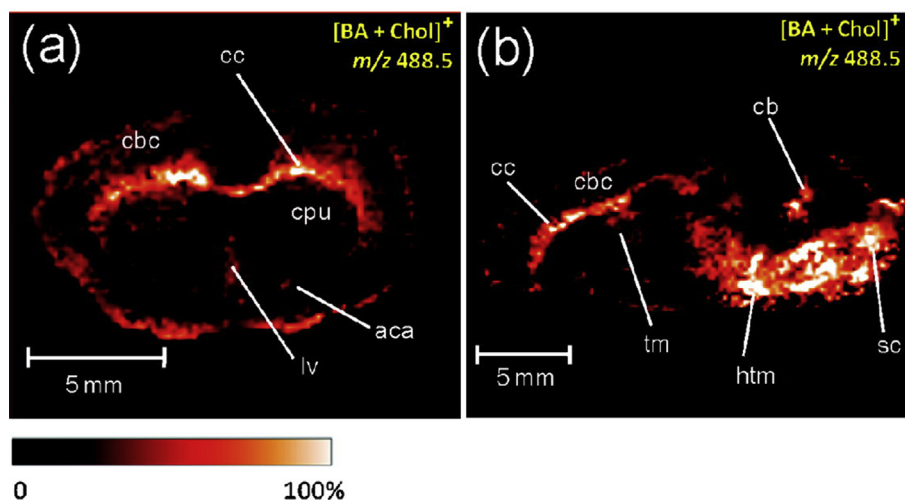


Fig. 7 – Localization of cholesterol sulfate in human skin tissue section. (a) H&E stained section, (b) MALDI-MSI imaging result for  $m/z$  465.3, (c) magnification of H&E stained section, (d) magnification of MALDI-MSI result. Reprinted with permission from Ref. [42].



**Fig. 8** – Localization of cholesterol in rat brain section observed by reactive DESI. Distribution of the signal at  $m/z$  488.5 in (a) coronal section and (b) sagittal section of rat brain. Reprinted with permission from Ref. [40].

the epidermis since it plays an important role in regulation of the barrier formation.

Compared to MALDI-MSI, DESI-MSI works under ambient conditions and therefore it allows interrogation of the sample in its native environment. It provides *in situ*, real time, and rapid mass spectrometric analysis. DESI-MSI does not require use of the matrix, allowing detection of low molecular weight species. Reactive DESI, incorporating betaine aldehyde (BA) into the DESI spray solvent to form easily ionized product (see Fig. 5b), was used for cholesterol imaging in rat brain with a spatial resolution of 200  $\mu\text{m}$  [40]. Signal at  $m/z$  488.5, corresponding to  $[\text{BA}+\text{Chol}]^+$  ion, had much higher intensity in the white matter as compared to the gray matter of rat brain (see Fig. 8).

In another study, DESI imaging was used to localize cholesterol sulfate in prostate cancer and normal tissue sections [22]. Cholesterol sulfate was identified as a differentiating compound between cancer and normal tissues. This compound was found almost exclusively in cancerous tissues, thus it is a promising biomarker candidate to serve as a tool for prostate cancer diagnosis.

## 6. Conclusions

Up to date, multiple analytical methods have been developed for analysis of cholesterol, including classical chemical methods, enzymatic assays, gas chromatography (GC), liquid chromatography (LC), and mass spectrometry (MS). Several golden standard methods have been established for clinical and industrial use. Nevertheless, new analytical platforms, such as ambient ionization mass spectrometry, are being developed and utilized. DART-MS was presented as a promising tool for rapid and cost-effective quantitation of cholesterol that could be possibly used in POCT. On the other hand, techniques such as MALDI-MSI and DESI-MSI were used to visualize spatial distribution of cholesterol and its derivatives

in tissue sections. Mass spectrometry-based molecular imaging will increase the understanding of physiological processes and disease pathology towards to a more precise absolute quantitation of molecules *in situ*.

## Conflicts of interest

None.

## Notes/Thanks/Other declarations

None.

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