


The importance of choosing the appropriate cholesterol quantification method: enzymatic assay versus gas chromatography

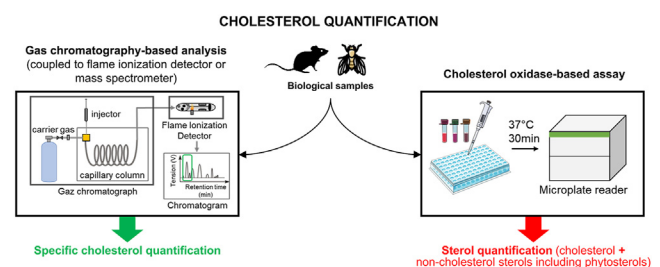
Jeanne Serrano^{1,2}, Lucy Martine¹, Yaël Grosjean² , Niyazi Acar¹, Georges Alves² , and Elodie A. Y. Masson¹ 

¹Eye & Nutrition Research Group, and ²Sensory Perception & Glia-Neuron Interaction Research Group, Centre des Sciences du Goût et de l'Alimentation, CNRS, INRAE, Institut Agro, Université de Bourgogne, Dijon, France

Abstract Cholesterol is a major lipid of the animal realm with many biological roles. It is an important component of cellular membranes and a precursor of steroid hormones and bile acids. It is particularly abundant in nervous tissues, and dysregulation of cholesterol metabolism has been associated with neurodegenerative diseases such as Alzheimer's and Huntington's diseases. Deciphering the pathophysiological mechanisms of these disorders often involves animal models such as mice and *Drosophila*. Accurate quantification of cholesterol levels in the chosen models is a critical point of these studies. In the present work, we compare two common methods, gas chromatography coupled to flame-ionization detection (GC/FID) and a cholesterol oxidase-based fluorometric assay to measure cholesterol in mouse brains and *Drosophila* heads. Cholesterol levels measured by the two methods were similar for the mouse brain, which presents a huge majority of cholesterol in its sterol profile. On the contrary, depending on the method, measured cholesterol levels were very different for *Drosophila* heads, which present a complex sterol profile with a minority of cholesterol. We showed that the enzyme-based assay is not specific for cholesterol and detects other sterols as well. This method is therefore not suited for cholesterol measurement in models such as *Drosophila*. Alternatively, chromatographic methods, such as GC/FID, offer the required specificity for cholesterol quantification.  Understanding the limitations of the quantification techniques is essential for reliable interpretation of the results in cholesterol-related research.

Supplementary key words sterol • Amplex® Red Cholesterol Assay kit • *Drosophila* • mouse

Cholesterol is a vital lipid presenting a unique structure composed of a sterol nucleus linked to a hydrocarbon chain and a 3 β -hydroxyl group. It is found in animals exhibiting a huge variety of functions. As a major component of cellular membranes, it plays a key role in



fluidity, ion permeability, and the organization of membrane domains. Beyond its structural functions, cholesterol is also a precursor of many bioactive molecules with signaling functions which are oxysterols and steroid hormones such as cortisol or progesterone in mammals and ecdysone in insects (1, 2). These diverse functions make cholesterol a major actor in numerous physiological processes and a potential player in various diseases. Especially, cholesterol is abundant in the nervous system where it supports neuron viability and transmission of the nervous signal (3), and disruption of cholesterol homeostasis has been linked to various neurodegenerative disorders, including Niemann-Pick type C disease, Alzheimer's disease (AD), and Huntington's disease (HD) (4). Various animal models have been used to better understand the role of cholesterol in the development of neurodegenerative diseases and the underlying mechanisms. For example, some studies using mouse models have demonstrated that an accumulation of cholesterol in the hippocampus and the striatum, due to impaired elimination, leads to typical features of AD and HD, respectively (5, 6). *Drosophila melanogaster* is another well-used model due to the accessibility of genome sequences, the simplicity of genetic manipulation, and the substantial homology with human disease-associated genes (7, 8). In *Drosophila*, mutation of

*For correspondence: Elodie A. Y. Masson, elodie.masson@inrae.fr; Georges Alves, georges.alves@u-bourgogne.fr.

the *Niemann-Pick type C1* gene (*Npc1*), coding for an intracellular cholesterol transporter essential for cholesterol metabolism, has shown the consequences of neuronal cholesterol deposits on age-dependent neurodegeneration (9).

In mammals, cholesterol is synthesized from acetyl-CoA via a series of enzymatic reactions generating many precursor molecules (Fig. 1A,C) (13, 14). On the other hand, *Drosophila* is a cholesterol auxotroph (2, 15) meaning that it cannot synthesize cholesterol de novo

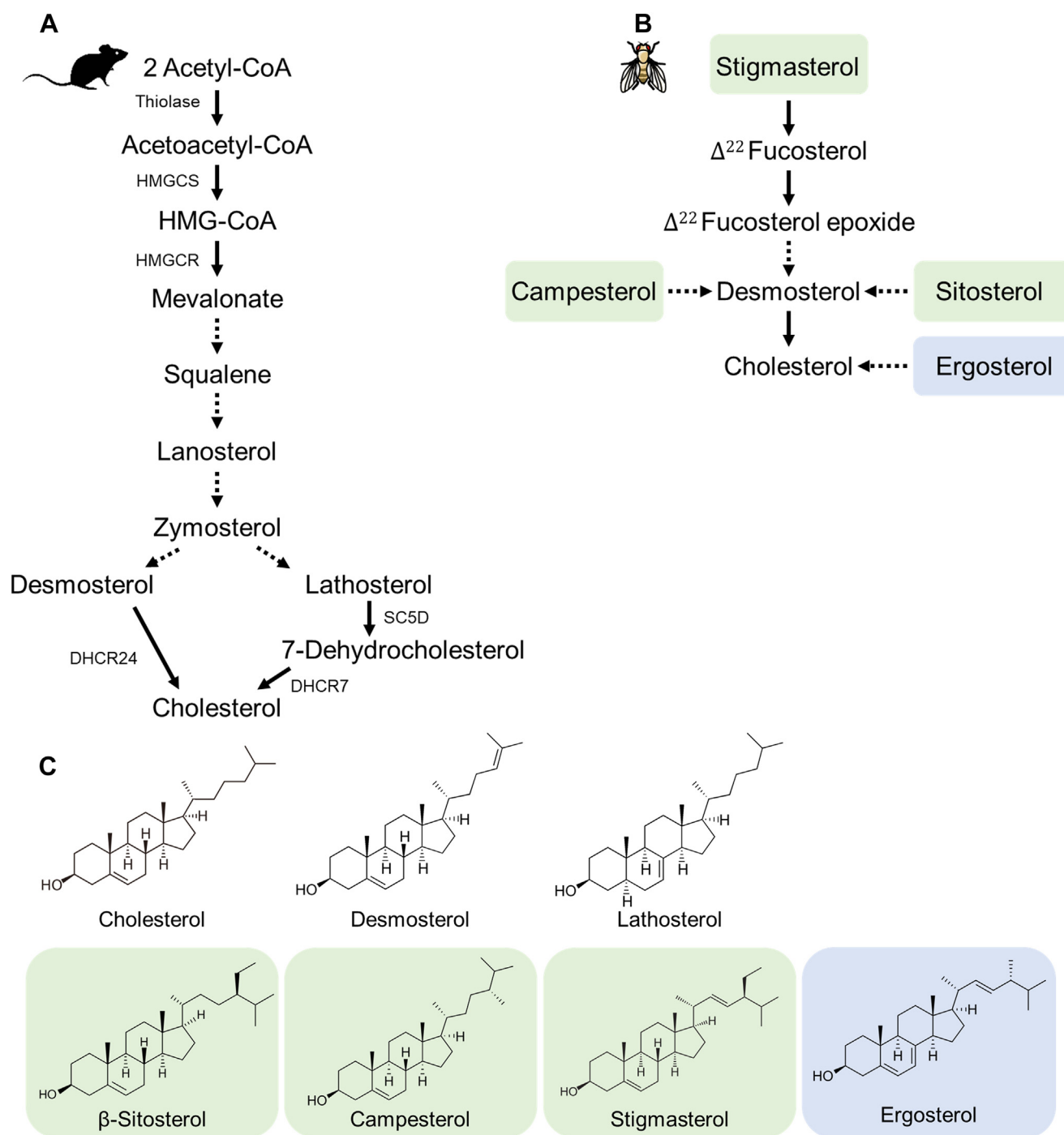


Fig. 1. Cholesterol synthesis in mouse and *Drosophila* and chemical structure of major sterols. A: In mice, a cascade of enzymatic reactions implicating HMGCS (hydroxymethylglutaryl-CoA synthase), HMGCR (hydroxymethylglutaryl-CoA reductase), DHCR (dehydrocholesterol reductase), and SC5D (sterol-C5-desaturase), converts two acetyl-CoA molecules into cholesterol. This process leads to the synthesis of sterols such as desmosterol and 7-dehydrocholesterol, which act as immediate precursors for cholesterol (10). Solid arrows indicate a direct step, while dotted arrows indicate several intermediate steps. B: In *Drosophila*, phytosterols such as stigmasterol, campesterol, and β -sitosterol undergo conversion to desmosterol, which serves as the direct precursor for cholesterol (11). A conversion pathway from ergosterol to cholesterol in arthropods was also proposed by Chamberlain *et al.* in 2004 (12). Implicated enzymes are still unknown. C: Each sterol is composed of a hydrophobic apolar core (steran nucleus linked to a hydrocarbon chain) and a polar hydrophilic head (β -hydroxyl group). Phytosterols are highlighted in green, while ergosterol derived from yeast is highlighted in blue.

and must acquire cholesterol or precursors from the diet. These dietary sources include plant (β -sitosterol, campesterol, stigmasterol) or yeast (ergosterol) sterols (Fig. 1B, C) (16, 17).

Reliable quantification of cholesterol levels in various experimental models and conditions is crucial. This is not trivial considering the large variety of compounds structurally closely related to cholesterol. Several methods are available such as enzymatic, chromatographic, or direct with different detection systems including fluorimetry or mass spectrometry. Current common methods are presented with a brief description in Table 1.

For the present study, we used two methods, namely gas chromatography coupled to flame ionization detection (GC/FID) or to mass spectrometry (GC/MS) and a fluorometric cholesterol oxidase-based assay, to quantify cholesterol in both mouse and *Drosophila* samples. Our results clearly demonstrated that the fluorometric enzymatic assay, exemplified by the Amplex® Red Cholesterol Assay (see Fig. 2 for a schematic representation), is not specific for cholesterol. Consequently, we do not recommend using this type of assay, without prior chromatographic separation of cholesterol, for cholesterol quantification in samples containing significant amounts of other sterols, as it is the case for *Drosophila melanogaster*.

MATERIALS AND METHODS

Reagents

Standards of cholesterol (C8667), 5 α -cholestane (C8003), ergosterol (45480), stigmasterol (S6126) and β -sitosterol (S1270) were bought from Sigma-Aldrich. Standards of campesterol (700126P) and desmosterol (700060P) were purchased from Avanti Polar Lipids. Chloroform (CHCl₃) and methanol (CH₃OH) were obtained from CARLO ERBA Reagents. BSTFA/TMCS (N,O-Bis(triméthylsilyl)trifluoroacetamide/trimethylchlorosilane) was purchased from Supelco (Merck).

Mice

C57Bl6J mice were housed in a controlled environment including temperature (22 \pm 1°C), hygrometry (55–60%) and light conditions (50 lux, 12 h light/dark cycles). Water and food were provided ad libitum, including a complete breeding diet comprising 5.1% lipids (#A03SP, Safelab).

All procedures were approved by the French Ministry of Higher Education and Research and conducted in accordance with the local ethics committee (Comité d'éthique de l'Expérimentation Animale Dijon Grande-Campus, University of Burgundy). At the age of 8–12 days, mice were sedated with isoflurane and sacrificed by decapitation to collect their brain. A hemisphere was dissected and snap-frozen in liquid nitrogen. These samples were subsequently stored at –80°C until used for experiments.

Fly stock

Flies (*Drosophila melanogaster*, Canton-S strain) were kept at 25°C with a 12 h light/dark cycle. *Drosophila* was raised on a

medium composed of brewer's yeast E50 (0.065 g/ml, MP Biomedicals), corn flour (0.065 g/ml, Eurogerm), agar (0.009 g/ml, Groupe MERIDIS, Montpellier, France), tegosept (3.1%, Genesee Scientific) and water. Seven-day-old flies were placed in 1.5 ml tubes and frozen in liquid nitrogen to make the tissues fragile and brittle. Tubes were then placed on a shaker for 30 s to separate the heads from the bodies by mechanical friction. The content of each tube was then transferred on two successive sieves, to retain the bodies, then the heads. 140 heads were collected and pooled to make a sample. Samples were stored at –80°C until used.

Lipid extraction

Tissues (140 *Drosophila* heads or a mouse half brain) were homogenized mechanically with tungsten microbeads in 1 ml of H₂O using a TissueLyser (Qiagen, The Netherlands, 1 min 30 s at 30 Hz speed). Before lipid extraction, 10% of the homogenate was taken for protein quantification. The remaining 90% were used for total lipid extraction according to Folch's procedure (40). In brief, the homogenate was combined with 10 ml of chloroform/methanol mixture (2:1, v/v). Following centrifugation (1,620 g, 3 min, room temperature (RT)), the lower organic phase containing lipids was collected. Total lipids were dried under a stream of nitrogen using a 35°C water bath and solubilized in 1 ml chloroform/methanol mixture (2:1, v/v). Finally, this lipid extract was divided for sterol analysis using either GC (10% of the lipid extract for mouse brains and 60% for *Drosophila* heads) or the cholesterol oxidase-based assay (40% for both samples).

Quantitative determination of protein content

The 10% aliquots of tissue homogenates were lysed by the addition of 5X Ripa buffer and incubated on ice for 30 min. After clearing by centrifugation (15,300 g, 10 min, 4°C), total protein content was measured on the supernatants using the Bicinchoninic acid (BCA) protein assay kit according to manufacturer's instructions (Pierce Biotechnology, Thermo Scientific) with bovine serum albumin as a standard (Sigma-Aldrich).

Sterol analysis using gas chromatography

The dedicated fraction of the total lipid extract was dried under a stream of nitrogen using a 35°C water bath. Total lipids were submitted to alkaline hydrolysis, and all sterols, that could not be saponified, were extracted. In brief, 5 ml of a 0.35 M potassium hydroxide solution in absolute ethanol was added to the dried lipids and agitated for 2 h at room temperature. Subsequently, 100 μ l of phosphoric acid, followed by 9 ml of chloroform and 3 ml of 0.73% sodium chloride, were added. After centrifugation (1,620 g, 3 min, RT), the lower phase containing sterols was collected, dried under a nitrogen stream using a 35°C water bath, and solubilized in a 1 ml chloroform/methanol mixture (2:1, v/v). In mouse samples, the total non-saponifiable material was divided for cholesterol (10%) and other sterols (90%) quantification. For *Drosophila* samples, the total non-saponifiable material was entirely used for the quantification of sterols altogether. The sterols were then derivatized into triméthylsilyl ether using BSTFA/TMCS (99:1, v/v) at 60°C for 30 min, after adding 1.2 μ g of 5 α -cholestane as a standard. Sterols were identified using gas chromatography coupled to a mass spectrometer (GC/MS) (5973N, Agilent) with a DB-5MS column of 30 m (length) x 0.25 mm (internal diameter) x 0.25 μ m (coating)

TABLE 1. Overview of the common analytical methods for cholesterol quantification

	Methods	Samples	Principles	Quantification Mode	References
Enzyme-based	Colorimetric or fluorometric-enzymatic assay (see Fig. 2)	Plasma/serum Food product Lipid extract from cells or tissues	<ul style="list-style-type: none"> - Detection of free or total (free + esterified) cholesterol via prior use of cholesterol esterase - Oxidation of cholesterol by cholesterol oxidase and production of H₂O₂ - Detection of H₂O₂ via a specific probe generating an absorbent or fluorescent compound 	Absorbance or fluorescence Cholesterol calibration curve (external standard)	(18–21)
Chromatographic	Gas chromatography coupled to flame ionization detection (GC/FID)	Lipid extract	<ul style="list-style-type: none"> - Detection of free or total (free + esterified) cholesterol via prior saponification and cholesterol extraction - Derivatization and separation of individual sterols on a polar column using a gaseous mobile phase after vaporization of the sample - Identification of sterols based on retention time compared with standards 	FID Cholesterol calibration curve Internal standard	(22–24)
	Gas chromatography coupled to mass spectrometry (GC/MS)	Lipid extract	<ul style="list-style-type: none"> - Detection of free or total (free + esterified) cholesterol via prior saponification and cholesterol extraction - Derivatization and separation of individual sterols on a polar column using a gaseous mobile phase after vaporization of the sample - Identification of sterols based on retention time and total ion chromatogram (profile of <i>m/z</i> of the molecule and its different ion fragments) compared with standards and sterol libraries 	MS Cholesterol calibration curve Isotopically labelled internal standard (² H or ¹³ C)	(25–28)
	High Performance Liquid chromatography coupled to mass spectrometry (LC/MS)	Lipid extract	<ul style="list-style-type: none"> - Detection of free or total (free + esterified) cholesterol via prior saponification and cholesterol extraction - Separation of individual sterols on a column using a solvent mobile phase - Identification of sterols based on retention time and total ion chromatogram (profile of <i>m/z</i> of the molecule and its different ion fragments) compared with standards 	MS Cholesterol calibration curve Isotopically labelled internal standard (² H or ¹³ C)	(29–31)
Direct	Matrix-assisted laser desorption ionization-time of flight (MALDI/MS)	Lipid extract Serum Tissue section	<ul style="list-style-type: none"> - Mixture of sample with a matrix - Application of the mixture to a sample plate - Identification of sterols based on total ion chromatogram (<i>m/z</i>) - Possibility of MS imaging 	MS Cholesterol calibration curve Internal standard	(32–34)
	Shotgun MS	Lipid extract	<ul style="list-style-type: none"> - Derivatization - Direct flow injection - Identification of sterols based on total ion chromatogram (<i>m/z</i>) 	MS Cholesterol calibration curve Isotopically labelled internal standard (² H or ¹³ C)	(17, 35)
	Nuclear magnetic resonance (NMR)	Plasma/serum Lipid extract	Identification of cholesterol based on the specific intrinsic spin properties of atomic nuclei exposed to an external magnetic field	NMR External or internal standard	(27, 36, 37)

GC/FID, gas chromatography/flame-ionization detection; GC/MS, gas chromatography/mass spectrometry; H₂O₂, hydrogen peroxide; MALDI/MS, Matrix-assisted laser desorption ionization/mass spectrometry; NMR, nuclear magnetic resonance.

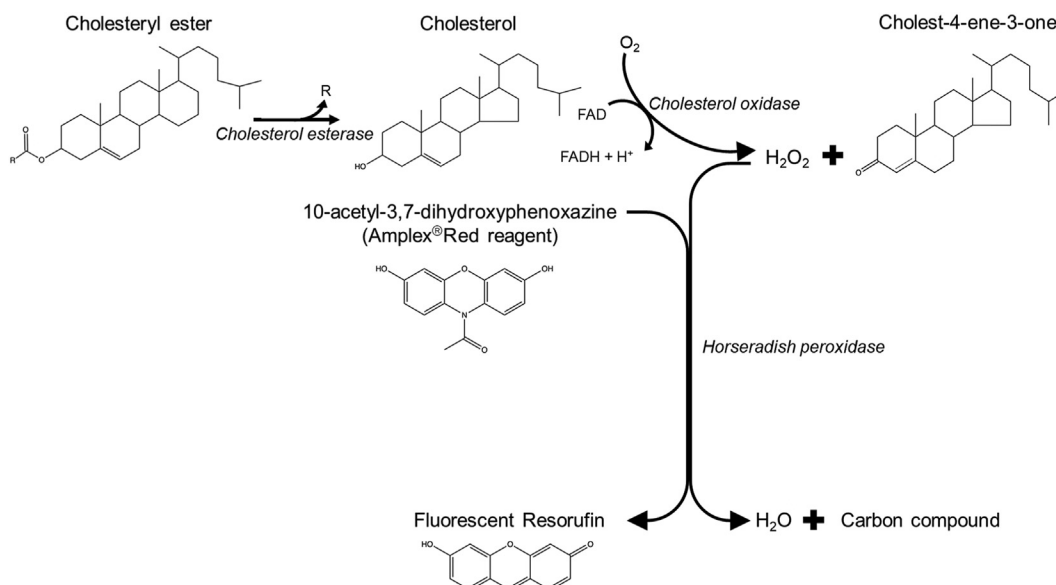


Fig. 2. Simplified sequential enzymatic reactions of the Amplex® Red Cholesterol Assay kit. This method relies on an enzyme-coupled process to measure both free cholesterol and esterified cholesterol. Cholesterol esterase separates fatty acid (represented by R group) from cholesterol, which is further converted into cholest-5-ene-3-one, consecutively isomerized into cholest-4-ene-3-one, and hydrogen peroxide (H₂O₂) by cholesterol oxidase. The produced H₂O₂ is detected using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex® Red reagent), a stable probe that produces fluorescent resorufin in the presence of horseradish peroxidase (38, 39).

(Agilent). Samples were injected using splitless mode at 290°C and separated under a temperature gradient as follows: 1 min 60°C, 20°C/min up to 290°C. Identification was performed in scan mode according to the retention times defined by unlabeled standards and specific spectra compared to the NIST bank (The NIST/EPA/NIH Mass Spectral Library Version 2.0f, build Oct 8 2008) and an internal base (41) (Supplemental Table S1). Quantification was performed using gas chromatography coupled to a flame ionization detector (GC/FID) (HP4890A, Hewlett-Packard). Conditions for injection and separation were similar to GC/MS and quantification was based on one-point calibration using 5 α -cholestane standard. This was conducted with six biological replicates and three technical replicates (3 GC injections for each biological replicate).

Sterol quantification using a fluorometric cholesterol oxidase-based assay

The Amplex® Cholesterol Assay kit (A12216 Thermofisher® scientific) was used to quantify cholesterol levels in the dedicated fraction of the total lipid homogenates. Mouse brain lipids were dried under a nitrogen stream using a 35°C water bath, resolubilized in 1 ml of ethanol/chloroform (9:1, v/v), and diluted 200 times in Amplex buffer. 10 μ l were loaded into white 96-well plates (nunc® Thermofisher® scientific) and Amplex Buffer was added to reach a total volume of 50 μ l. *Drosophila* heads' lipids were dried under a nitrogen stream using a 35°C water bath, resolubilized in 100 μ l of ethanol/chloroform (9:1, v/v), and diluted 100 times in Amplex Buffer. 50 μ l of the solution were added to the microplate well. This procedure was carried out for six biological replicates. In addition, three measurements (technical replicates) were done for each biological replicate. In parallel, several synthetic lipid standards were used, namely 5 α -cholestane, cholesterol, ergosterol, campesterol, stigmasterol, β -sitosterol, and desmosterol. Sterol standard solutions were prepared in a chloroform/methanol

mixture (2:1, v/v). Subsequently, dried standards were solubilized in Amplex buffer to a final concentration of 1.2 μ M. 50 μ l were loaded into wells in triplicate. The assay was then performed according to the manufacturer's instructions. Briefly, 50 μ l of a solution containing the enzymes (cholesterol oxidase, horseradish peroxidase (HRP) and cholesterol esterase) and the H₂O₂ probe (Amplex® Red reagent) were added to each well. After incubation at 37°C for 30 min, fluorescence was measured at an excitation wavelength of 570 nm and an emission wavelength of 590 nm, using the Ensign Multimode Plate Reader instrument (PerkinElmer). Specific cholesterol standard curves containing 1% and 0.5% ethanol/chloroform (9:1, v/v) were used for quantification of *Drosophila* and mouse samples, respectively.

Statistical analysis

Normality was tested using the Shapiro–Wilk test. Results were submitted to Mann–Whitney *U* test to perform a non-parametric statistical analysis using GraphPad Prism9 software (GraphPad Software). Data are represented as the median, interquartile range (IQR), or mean \pm SEM depending on the figure. Statistical significance is indicated as **P* < 0.05 and ***P* < 0.01.

RESULTS

GC analysis reveals species-specific sterol profiles

Sterols of the mouse brain and *Drosophila* heads were analyzed using GC, which enabled the separation of the individual sterols present in the samples. First, those were identified using MS by comparison with databases. Then, their levels were quantified by coupling to a flame ionization detector (GC/FID) thanks to the use of 5 α -cholestane as a standard using

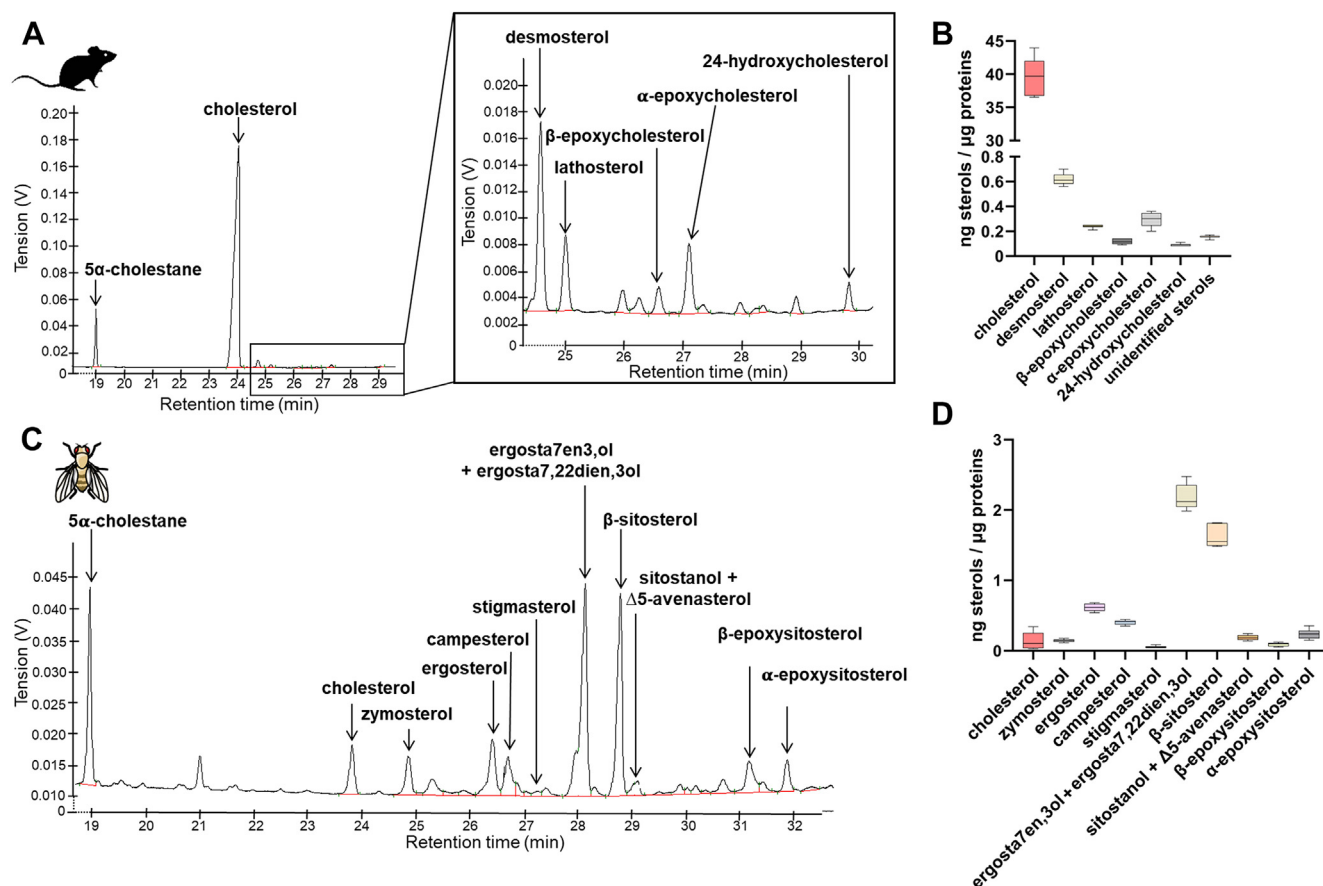


Fig. 3. GC/FID sterol profiles of mouse brain and *Drosophila* heads. A representative GC/FID chromatogram illustrates the sterol profile of the mouse brain (A) and *Drosophila* heads (C). Sterols were identified using GC/MS in scan mode according to their retention time defined by unlabeled standards and specific spectra compared to the NIST bank (The NIST/EPA/NIH Mass Spectral Library Version 2.0f, build Oct 8 2008) and an internal base (41). All sterols were quantified (B–D) using 5 α -cholestane as a standard. Ergosta7en,3ol and ergosta7,22dien,3ol as well as sitostanol and Δ 5-avenasterol could not be separated using our chromatographic conditions and were quantified together. Data are presented as median \pm min and max values of 6 biological replicates. Each biological replicate was injected 3 times.

one-point calibration. As shown in Fig. 3, cholesterol was by far the most abundant sterol in mouse brains (39.7 ng/ μ g proteins, IQR = 36.8–42.0), accounting for 96% of the total sterols detected. Desmosterol and lathosterol, two cholesterol precursors, were also present, at lower levels (0.61 ng/ μ g proteins, IQR = 0.58–0.66 and 0.24 ng/ μ g protein, IQR = 0.23–0.25 respectively). A metabolite of cholesterol called 24-hydroxycholesterol could also be detected and quantified in mouse brains. While epoxycholesterols were quantified, their relatively high amounts suggest that some degree of autooxidation occurred as an artifact during the procedure. Conversely, *Drosophila* heads contained a variety of sterols, the most abundant ones being ergosterol-related compounds (ergosta7en,3ol and ergosta7,22dien,3ol) (2.12 ng/ μ g proteins, IQR = 2.05–2.36) and β -sitosterol (1.55 ng/ μ g proteins, IQR = 1.49–1.81), accounting for 38% and 29% of total sterols, respectively. Interestingly, cholesterol levels were very low in *Drosophila* heads (0.10 ng/ μ g proteins, IQR = 0.04–0.25) compared with mouse

brains, accounting for only 2.5% of total sterols. Other sterols were also measured, such as ergosterol (0.62 ng/ μ g proteins, IQR = 0.57–0.67), campesterol (0.41 ng/ μ g proteins, IQR = 0.38–0.43), stigmasterol (0.05 ng/ μ g proteins, IQR = 0.04–0.06), Δ 5-avenasterol and sitostanol (0.18 ng/ μ g proteins, IQR = 0.16–0.22).

Cholesterol levels measured by GC/FID and the cholesterol oxidase-based assay are different in *Drosophila* heads

Mouse and *Drosophila* samples underwent measurement using a fluorometric cholesterol oxidase-based assay, namely the Amplex® Red Cholesterol Assay kit. Results were compared to the levels of cholesterol and total sterols, corresponding to the sum of all sterols quantified, using GC/FID (Fig. 4). In the mouse brain, the levels measured by the fluorometric enzyme-based assay (35.68 ng/ μ g proteins, IQR = 33.59–37.82) were close to the cholesterol levels measured using GC/FID (39.7 ng/ μ g proteins, IQR = 36.8–42.0). Those levels were also close to the total sterol levels measured by

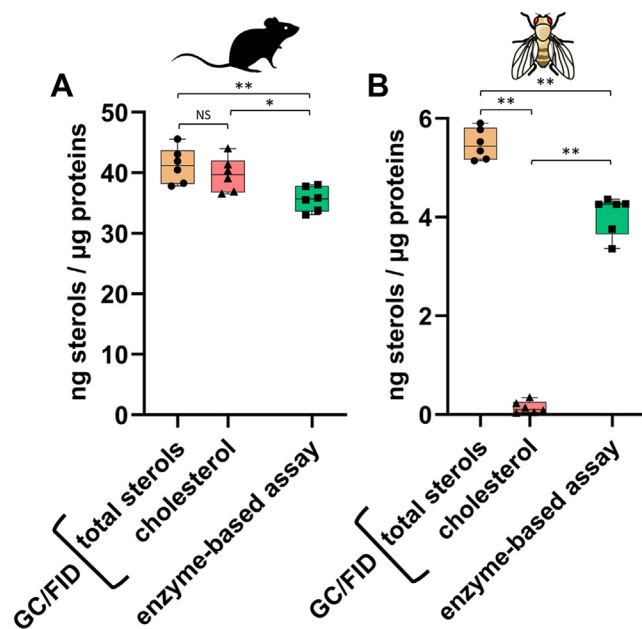


Fig. 4. Sterol levels measured using GC/FID and a fluorometric cholesterol oxidase-based assay in mouse brain and *Drosophila* heads. Both methods were employed to quantify sterols in lipid extracts of mouse brain (A) and *Drosophila* heads (B). Total sterol levels correspond to the sum of all sterols quantified by GC/FID. Data are represented as median \pm min and max values of 6 biological replicates represented by circles, triangles or squares. * $P < 0.05$; ** $P < 0.01$ (Mann-Whitney U test).

GC/FID (41.19 ng/ μ g proteins, IQR = 38.17–43.68) since cholesterol is the major sterol in the mouse brain. On the contrary, in *Drosophila* heads, the levels measured by the fluorometric enzyme-based assay (4.26 ng/ μ g proteins, IQR = 3.66–4.29) were considerably higher than the cholesterol levels measured by GC/FID (0.10 ng/ μ g proteins, IQR = 0.04–0.25) and were closer to the total sterol levels (5.44 ng/ μ g proteins, IQR = 5.17–5.81). Since *Drosophila* heads contain many sterols and a minority of cholesterol, these data suggest that the fluorometric cholesterol oxidase-based assay quantifies not only cholesterol but other sterols as well.

The fluorometric cholesterol oxidase-based assay is not specific for cholesterol

In order to assess the specificity of the fluorometric cholesterol oxidase-based assay, we tested its ability to detect and quantify several sterol standards. The signal produced by the various standards using the Amplex® Red Cholesterol Assay kit was measured and compared to the one produced by cholesterol. Our results clearly showed that this type of assay can detect and quantify several sterols with varying degrees of response (Fig. 5). Strikingly, the enzymatic assay was twice as responsive to desmosterol as it was to cholesterol (with a ratio of fluorescence intensity (FI) sterol/FI cholesterol = 2.30, IQR = 2.30–2.33). Among the tested sterols, the enzyme-based assay also detected campesterol, β -sitosterol, and ergosterol but half as efficiently as it did for cholesterol. The

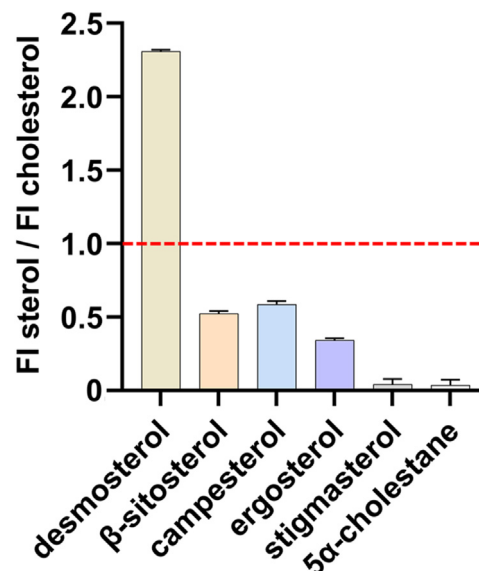


Fig. 5. Sterol standards measured using the cholesterol oxidase-based assay. Equimolar concentration of various sterol standards (1.2 μ M) were measured using the enzyme-based Amplex® Red Cholesterol Assay kit. For each sterol, results are presented as the ratio of the fluorescence intensity (FI) of the sterol considered to the FI of cholesterol (represented by a dotted red line). 3 technical replicates were done. Data are presented as mean \pm SEM.

signal detected for stigmasterol was very weak compared to the other sterols. Of note, we confirmed the purity of all sterol standards used except ergosterol, which contained 4.4% of cholesterol and 9.6% of another unidentified compound. 5 α -cholestane, which does not contain the hydroxyl group present in sterol compounds, was not quantified using this method. Overall, these observations demonstrate that the cholesterol oxidase-based assay is not specific for cholesterol.

DISCUSSION

Considering the large diversity of sterol compounds, structurally closely related, exhibiting various functions and abundance depending on the experimental model, specific quantification of cholesterol is both a requirement and a challenge. Several cholesterol quantification techniques are currently available (see Table 1) and it is crucial to understand their principles and limitations to avoid result misinterpretations.

In the present study, we compare two common cholesterol quantification methods, namely GC/FID and the Amplex® Red Cholesterol Assay kit, to quantify cholesterol in two different types of samples: mouse brain and *Drosophila* heads. We selected this kit because it is widely used in both vertebrate and *Drosophila* communities (38, 42). However, several other kits using the same methodology are commercially available from different providers. These enzyme-based assays are known for their sensitivity, ease of use, and the advantage of necessitating no expensive equipment.

First, by employing GC/FID, we conducted an analysis of the sterol profile. In mouse brains, cholesterol constitutes the large majority of sterols, with the remaining quantified sterols being cholesterol precursors, epoxysterol, and 24-hydroxycholesterol, consistent with previous findings (43, 44). The cholesterol content of the mammalian brain relies on endogenous de novo synthesis since the blood–brain barrier does not permit the entry of cholesterol from the circulation. However, it has been shown that circulating plant sterols can enter the brain and that feeding mice with a plant sterol-enriched diet leads to increased levels of phytosterols in the brain (45). We did not detect phytosterols in the brains of our mice. In contrast, *Drosophila* heads exhibit a sterol profile with a diverse range of sterols, in which cholesterol represents only a small fraction, as it has already been shown (16, 17). The predominant sterols identified in *Drosophila* heads are ergosterol-related compounds, along with β -sitosterol. Additionally, other phytosterols such as campesterol, Δ^5 -avenasterol, and stigmasterol are also present. In this cholesterol auxotroph insect, dietary sterols are absorbed from the midgut epithelium by the NPC1b protein and then transported into the hemolymph up to the various organs through lipophorins (46, 47). As a result, the sterol profile is highly dependent on the diet's richness in specific phytosterols. Indeed, previous studies quantified sterols in different organs of flies reared on yeast-based or plant-based food by using MS analyses (16, 17). The head of *Drosophila* reared on yeast-based food predominantly contained ergosterol, whereas those reared on plant-based food contained mainly β -sitosterol, followed by stigmasterol and campesterol. Furthermore, Carvalho *et al.* demonstrated that adding ergosterol or stigmasterol to the diet results in a notable elevation of these sterols in the brain (16). In our study, flies were reared on a combination of plant and yeast food (see Supplemental Fig. S1), explaining the variety of the sterol profile observed in *Drosophila* heads.

Then, we used a cholesterol oxidase-based assay and compared the quantified amounts with GC/FID. In a mouse brain, in which cholesterol is the primary sterol present, the levels detected using the enzyme-based assay were close to both the cholesterol levels and the sum of total sterols measured by GC/FID. On the contrary, in *Drosophila* heads, in which cholesterol is a minor sterol, the levels detected using the enzyme-based assay were drastically higher than the cholesterol levels measured by GC/FID. These findings suggested that the fluorometric cholesterol oxidase-based assay lacks cholesterol specificity. We confirmed this by showing that this assay can detect and quantify various sterol standards with varying degrees of response. The relative signal intensity was two-fold higher for desmosterol and two-fold lower for β -sitosterol, campesterol, and ergosterol compared with cholesterol. This results from the fact that the cholesterol oxidase used in this assay is not specific for


cholesterol (48). Indeed, the cholesterol oxidase from *Streptomyces* has been reported to oxidize many β -hydroxysteroids among which cholesterol but also 5α -cholestanol (5α -cholestan- β -ol), pregnenolone (pregn-5-en- β -ol-20-one), or epiandrosterone (5α -androstane- β -ol-17-one). The relative oxidation rates varied from 1 to 91% compared with cholesterol. No phytosterol was tested in this study. Steroids lacking the β -hydroxyl group were resistant to enzymatic oxidation, which explains why we did not detect 5α -cholestane when tested as a standard in the cholesterol oxidase-based assay. (49). In another study, the activity of the cholesterol oxidase from several micro-organisms (*Brevibacterium sterolicum*, *Rhodococcus equi*, and *Streptomyces hygroscopicus*) was tested toward different substrates, such as ergosterol, β -sitosterol, 7α or 7β -hydroxycholesterol, 7 keto-cholesterol, cholestanol, and pregnenolone. Pollegioni *et al.* thus demonstrated that the activity of the enzyme varied from 13% to 119% relative to cholesterol (50). The various oxidation rates observed in these two studies and the present indicate that the side chain which differs between the different sterols considered is able to modulate the degree of oxidation a specific sterol can undergo, possibly by modulating the accessibility of the sterol molecule to the reactive center of the enzyme. This could explain why we did not detect stigmasterol, despite the presence of the β -hydroxyl group in the ring structure. The importance of the side-chain structure is supported by the work by Slotte *et al.* which showed that the rate of oxidation increased with the length of the C17 side-chain in lipid monolayers and unilamellar vesicles (51). Moreover, Jove *et al.* highlighted another type of issue using the same enzyme-based assay while working in the field of cholesterol intestinal absorption. They observed that the presence of food extracts of vegetable origin (cocoa and tea-derived) induced interferences in the chain of enzymatic reactions. These included the cholesterol-independent generation of H_2O_2 due to an interaction of antioxidants present in the extracts with HRP and inhibition of cholesterol-oxidase activity in the presence of bile. The authors recommended the use of a cholesterol-independent condition as a blank and of cholesterol standard curves containing the extracts at working concentrations (52).

Overall, it appears that the cholesterol oxidase-based assay can generate a fluorescence signal nonspecific for cholesterol and consequently leads to inaccuracies when used for cholesterol quantification. Accurately, this type of assay should rather be named a sterol oxidase assay. Nevertheless, commercially available cholesterol oxidase-based assays are still widely used to quantify cholesterol in various samples. This is not a major issue in most mammalian samples, such as human plasma or mammalian blood, cellular or tissue extracts, in which cholesterol is by far the most abundant sterol. However, it might be problematic in specific conditions such as *Abcg5* or *Abcg8* knock-out mice or animals under a plant sterol-enriched diet or in patients with sitosterolemia (45, 53). It

is also highly problematic in samples containing a complex mixture of sterols with cholesterol being a minor component, such as *Drosophila*. In this latter case, specific differences in cholesterol content might not be detected using a cholesterol oxidase-based assay because of the presence of larger amounts of other sterols also metabolized by the enzyme. Alternative methods are available for precise and specific cholesterol quantification. Those involve the use of chromatography which enables the prior separation of cholesterol from other sterols that can interfere with its detection and quantification thus offering specificity. Those include GC coupled to FID or MS as used in the present work or other chromatographic methods such as HPLC or thin layer chromatography which can be used to separate cholesterol prior to an enzymatic assay. Moreover, the use of internal standards further improves accuracy in cholesterol quantification. The need for standardization in cholesterol measurement was highlighted by an original survey by Lütjohann and collaborators about 10 years ago. By comparing cholesterol and other sterol levels quantified by twenty laboratories across Europe on the same serum samples by GC/FID, GC/MS or LC/MS, they showed high interlaboratory variation despite the use of comparable methodologies and standardized material (54). In the present study, it is noticeable that the cholesterol levels measured by GC/FID are close but significantly higher than the levels measured by the enzyme-based assay in mouse samples despite the almost exclusive presence of cholesterol. This is not that surprising because the two methods rely on very different physicochemical principles. Additionally, visualizing cholesterol distribution within tissues can be achieved through on-tissue derivatization followed by MS imaging. This approach has been applied successfully to quantify cholesterol in different regions of the mouse brain, revealing, for example, a deficiency in cholesterol in hypomyelinated fiber tracts in *Npc1*^{-/-} mice (55). This mapping was accomplished using MALDI/MSI alongside the analysis of other sterols and oxysterols in the mouse brain (56). While these methods have been employed in *Drosophila* for lipid species analysis, no cholesterol map for the *Drosophila* brain seems to have been generated yet (57).

In conclusion, we clearly show in the present study that cholesterol-oxidase-based assay is not specific for cholesterol and therefore not appropriate for cholesterol quantification in sample types containing significant amounts of other sterols.

Data Availability

Sterol quantification raw data will be shared upon request: Elodie A.Y. Masson, INRA-UMR CSGA, 17, rue Sully, BP 86510, 21065, Dijon, France E-mail address: elodie.masson@inrae.fr. 

Supplemental data

This article contains [supplemental data](#).

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Author contributions

J. S., G. A., E. A. Y. M. conceptualization; J. S. and E. A. Y. M. formal analysis; J. S., L. M., investigation; J. S., L. M., and E. A. Y. M. methodology; J. S., L. M., and E. A. Y. M. validation; J. S. and E. A. Y. M. visualization; J. S. and E. A. Y. M. writing—original draft; J. S., L. M., Y. G., N. A., G. A., and E. A. Y. M. writing—review & editing; Y. G., N. A., G. A., and E. A. Y. M. funding acquisition; Y. G., N. A., G. A., and E. A. Y. M. supervision; G. A. and E. A. Y. M. project administration.

Author ORCIDs

Yael Grosjean  <https://orcid.org/0000-0003-0689-8344>

Georges Alves  <https://orcid.org/0000-0002-5548-0270>

Elodie A.Y. Masson  <https://orcid.org/0000-0001-6312-0913>

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

AD, Alzheimer's disease; BCA, Bicinchoninic acid; DHCR, dehydrocholesterol reductase; FI, fluorescence intensity; GC/FID, gas chromatography/flame-ionization detection; GC/MS, gas chromatography/mass spectrometry; H₂O₂, hydrogen peroxide; HD, Huntington's disease; HMGCR, hydroxymethylglutaryl-CoA reductase; HMGCS, hydroxymethylglutaryl-CoA synthase; HRP, horseradish peroxidase; IQR, interquartile range; MALDI/MS, matrix-assisted laser desorption ionization/mass spectrometry; NMR, nuclear magnetic resonance; *Npc1*, *Niemann-Pick type C1* gene; RT, room temperature; SC5D, sterol-C5-desaturase.

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