



LC–MS Approaches for Oxysterols in Various Biosamples

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Abstract

Oxysterols are involved in a plethora of biological processes, including a wide variety of diseases. Therefore, monitoring oxysterols is important for obtaining a deeper understanding of their biological roles and utilizing them as, for example, biomarkers. However, oxysterols can be challenging compounds to study, as they can be very similar in chemical structure but still have distinct biological roles. In addition, oxysterols may be difficult to detect, even with advanced analytical instrumentation. We here focus on the use of liquid chromatography–mass spectrometry (LC–MS) for the analysis of oxysterols, with an additional focus on the steps needed to prepare oxysterols for LC–MS. Steps can include chemical modification of the oxysterols for improving LC–MS sensitivity and adding chemicals that can reveal if the oxysterol levels have been perturbed during preparation. We then round off with descriptions and applications of various sample preparations for different biological matrices, from blood to cells, and biosamples with emerging attention, for example, exosomes and organoids. Taken together, oxysterol analysis is highly compatible with a wide variety of biosamples,

allowing for a deeper understanding of these challenging analytes.

Keywords

Oxysterols · Biosamples · Liquid chromatography–mass spectrometry · Derivatization · Sample preparation · Exosome · Organoids

Abbreviations

APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
BHT	Butylated hydroxytoluene
CZE	Capillary zone electrophoresis
DESI	Desorption electrospray ionization
EADSA	Enzyme-assisted derivatization for sterol analysis
ER–	Estrogen receptor negative
μLESA	Microscale liquid extraction for surface analysis
ER+	Estrogen receptor positive
ESI	Electrospray ionization
GC	Gas chromatography
GPCR	G protein-coupled receptors
LC	Liquid chromatography
LC–MS	Liquid chromatography–mass spectrometry
LXR	Liver X receptor

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<i>m/z</i>	mass-to-charge ratio
MALDI	Matrix-assisted laser desorption ionization
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSI	Mass spectrometry imaging
NAFLD	Nonalcoholic fatty liver disease
RPLC	Reversed phase liquid chromatography
SFC	Supercritical fluid chromatography
SMO	Smoothened
TMS	Trimethylsilyl
UHPLC	Ultra-high pressure liquid chromatography

4.1 Introduction

Oxysterols are metabolites of cholesterol that are formed enzymatically (e.g., by CYP450 enzymes) or by autoxidation. A variety of oxysterols exist (for some examples of oxysterols and their pathway, see Fig. 4.1) with various biological activities, for example, as ligands of nuclear receptors such as the liver X receptor (LXR) and estrogen receptor (ER), G protein-coupled receptors, and smoothened (Griffiths and Wang 2019). Oxysterols are involved in a plethora of diseases, such as cancer, Epstein–Barr, nonalcoholic fatty liver disease (NAFLD), Alzheimer’s disease, and others. Therefore, the identification and quantification of the different oxysterol isomers are important for understanding the biological mechanisms behind the diseases and for biomarkers/diagnostics. The oxysterols can be present in biological samples as free sterols (nonesterified) or conjugated to, for example, a fatty acid (esterified).

The analysis of oxysterols can be challenging. For example, many oxysterols with distinct biological roles are structural isomers (e.g., different hydroxycholesterols or dihydroxycholesterols). Mass spectrometry (MS) is often the method of choice for analyzing biosamples, but for oxysterols, this is far from straightforward. For example, the aforementioned isomers (see also Fig. 4.1) share the same mass-

to-charge ratio (m/z) and will then result in identical signals in the mass spectrometer; this can even be the case when performing tandem MS (MS/MS), which essentially consists of fragmenting the parent mass and applying MS to the resulting fragments. This lack of distinct analytical features becomes a bottleneck for distinguishing compounds with different biological and diagnostic roles. To secure the identification of the different isomers, a separation step upstream of the MS analyzer is often needed. This separation step is very often based on chromatography, for example, liquid chromatography (LC), gas chromatography (GC), or supercritical fluid chromatography (SFC, also known as convergence chromatography, UPCC). In addition, other separation approaches can be utilized, such as capillary zone electrophoresis (CZE). In chromatography separation, a sample is driven by a mobile phase through a column that contains an immobilized stationary phase. The mobile phase can be a liquid (LC), gas (GC), or supercritical fluid (SFC). For CZE, the solvent is an electrolyte but does not have a stationary phase (and is hence not defined as a type of chromatography but an electrophoretic approach). The different components in a sample are separated based on their different degrees of affinity for a stationary phase, based on, for example, polarity, charge, or size, as they are driven through the column with the mobile phase. For example, one compound in a sample that has little affinity for a stationary phase is poorly retained by the column and will hence exit the column before a compound that has a stronger affinity for the stationary phase and is more retained. Compounds are then separated and enter the mass spectrometer at different time points, called retention times.

Traditionally, oxysterol separations have been performed by GC after a derivatization step to make the oxysterol volatile enough for GC separation (a major prerequisite but also a limitation of GC analysis). Typically, oxysterols are derivatized with trimethylsilyl. The derivatization steps most often include an alkaline hydrolysis step, allowing the determination of total oxysterol content (nonesterified and esterified). The

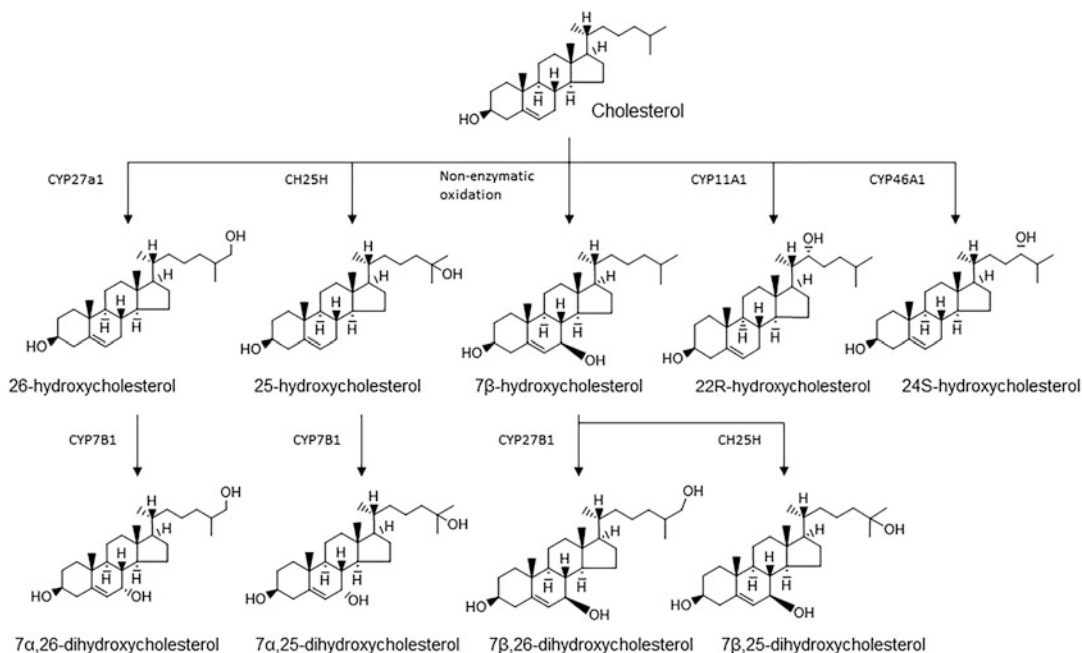


Fig. 4.1 Structure of some oxysterol isomers and the precursor cholesterol. All the hydroxycholesterols (and also the dihydroxycholesterols) have the same molecular

mass, resulting in the same signal in the MS. A separation, for example, by chromatography, is important for correct isomer identification

hydrolysis and derivatization steps are often performed at high temperatures, which might cause the autoxidation of cholesterol (the precursor) into oxysterols during the sample preparation (see textbox 1), creating false positive results. In addition, GC can have limitations regarding limited samples, and derivatization steps may not always be applicable to all oxysterol types. Other separation approaches may have limitations. For example, a CZE separation is largely dependent on the charge and hydrodynamic size of the compounds that are to be separated, which may also be very similar for many oxysterols. SFC is limited to compounds that have solubility in supercritical fluids, which can also be a limiting factor for several oxysterol variants, both known and unknown. LC is, however, arguably a more versatile approach, as it is not dependent on elevated temperature or analyte volatility and enjoys a host of separation principles suited for the wide range of oxysterol variants. In addition, LC is a current go-to approach for the separation of very limited

samples, for example, metabolites and proteins, even down to single cells and beyond (Røberg-Larsen et al., 2021). Due to these reasons, we here focus on LC-MS approaches for analyzing oxysterols in various biosamples. First, we describe some general aspects of the separation and detection of oxysterols with LC-MS, followed by considerations of whether one wants to analyze specific target oxysterol analytes or undertake a more global approach. We then continue with a focus on how the sample cleanup is done for securing sensitive analysis of oxysterols in biological samples, and finally, we discuss how these parts are applied for analysis of different types of samples, such as cells, exosomes, tissues, and blood.

Autoxidation

Stemming from their precursor cholesterol, oxysterols are formed by enzymatic oxidation or nonenzymatic oxidation

(continued)

(autoxidation). Although autoxidation is controlled in a biological system, it may be less straightforward to control during the time from sample preparation to analysis as the oxysterols are present in low concentrations in our body against a high background of the precursor cholesterol. For example, cholesterol in the samples can be oxidized into oxysterols at elevated temperatures. Autoxidation artifacts cannot be separated from oxysterols; hence, they are quantified as oxysterols. This might be one of the reasons for the large variation in oxysterol concentration reported in biosamples between studies (the mass spectrometer cannot distinguish between an oxysterol produced in the body and one unwantingly created during sample handling). Cholesterol can be removed from the sample early in the sample handling process (e.g., by solid-phase extraction). However, such steps may come with the potential of losing oxysterols as well, compromising the sensitivity of the method (not unlike proteins that may be lost when performing a depletion of the most abundant proteins in a blood proteomics sample). Other approaches, such as adding heavy cholesterol to the sample for autoxidation monitoring, are also possible. If cholesterol is oxidized during sample preparation, the heavy cholesterol is oxidized to heavy oxysterols, which can be monitored in the MS. This will not remove the autoxidation problem, but samples that have proven autoxidation through the monitoring of heavy oxysterols can be discarded. Even with approaches as described here, autoxidation is a key concern one must have during the monitoring of oxysterols and the development of novel methodologies.

4.2 LC–MS of Oxysterols

4.2.1 How Are Oxysterols Separated?

In liquid-based separations, the most common separation principle is reversed-phase LC (RPLC). In RPLC, the stationary phase is hydrophobic (e.g., alkyl chains covalently bound to porous silica particles) and the mobile phase is polar, consisting of water and a water-mixable organic solvent, for example, methanol or acetonitrile. A buffer or an acid is added to the mobile phase for pH control. Separation is based on the different degrees of hydrophobic interactions between the analytes and the stationary phase. RPLC is often the preferred separation technique when handling biosamples due to its robustness and high compatibility with aqueous biosamples and MS.

The separation step is, as mentioned, of high importance when isomers are to be detected and measured. Most separations of oxysterols are performed using octadecyl alkyl chain (C18)-bonded silica stationary phases. With RPLC, more polar oxysterols elute first, for example, dihydroxy cholesterol, followed by more hydrophobic side-chain hydroxylated and ring-hydroxylated sterols. Different C18 columns might provide somewhat different retention and selectivity of the isomers depending on, for example, the carbon load of the column and modifications to the stationary phase chemistry. The choice of organic modifier in the mobile phase (methanol, acetonitrile, or combinations of several organic solvents) also affects the selectivity of the separation. For a comprehensive comparison of columns for derivatized and nonderivatized oxysterol determination, see Dias et al. (2018b).

4.2.2 How Are Oxysterols Transferred to the Mass Spectrometer?

To transfer the analytes (often thermolabile) from the liquid phase into charged gas-phase ions (the form in which compounds are analyzed by MS), a

transitional step is needed between the LC and MS. The most common approach is applying electrospray ionization (ESI). Other ionization sources for LC–MS, for example, atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI), have also been successfully applied for oxysterol analysis, but ESI is by far the most widely used. ESI is essentially placing an electric potential between the LC outlet and the MS inlet. Instead of solvent dripping out of the LC columns, the applied voltage generates charged droplets that fly toward the MS inlet. Due to the surplus charge of the droplets, the repulsion results in the droplets exploding into smaller droplets, reaching the final stage where analytes dissociate from the droplets into gas phase ions without the aid of temperature. This process requires that the analytes be charged/chargeable. Oxysterols are, due to their neutral nature, not ESI-friendly. Therefore, various approaches such as derivatization to enhance detection sensitivity have been applied, for example, enzyme-assisted derivatization for sterol analysis (EADSA) with Girard P or T, picolinyl ester, or *N,N*-dimethylglycine esters. The common goal for all these approaches is to incorporate a charge or chargeable group into the oxysterol to enhance sensitivity when applying ESI. In addition, most of these derivatization approaches result in MS/MS fragmentations that are highly specific, further enhancing sensitivity and identifying (new) oxysterols. For a more comprehensive review of derivatization strategies for LC–MS determination of oxysterols, see, for example, Griffiths et al. (2016). Most of these derivatization reactions are performed at ambient temperature, decreasing the risk of autoxidation of cholesterol into oxysterols during sample preparation compared to GC derivatization reactions.

Together with the development of higher-sensitivity MS instruments, LC–MS determination of oxysterols without derivatization is possible. McDonald et al. (2012) created adducts with ammonium, charging the oxysterols (and other sterols), resulting in detection levels of 1 ng/mL from 200 μ L of plasma. This approach is highly instrument-dependent (e.g., dependent on ionization source temperature; Mendiara et al.

2018). In contrast to many GC-based approaches, most derivatization strategies for LC–MS allow for either free (nonesterified) or total (esterified and nonesterified) quantification of oxysterols.

Most MS instruments are solely based on measuring the mass-to-charge (m/z) ratio of compounds and their fragments, including both high-resolution units such as time-of-flight mass spectrometers and triple quadrupole mass spectrometers, which have a lower resolution but traditionally superior quantitative traits. We have here set a premise that MS is dependent on a chromatography-related step. However, there are variants of MS that can assist with the separation of compounds, for example, ion mobility MS. Ion mobility MS is increasingly used in bioanalysis, both in proteomics and metabolomics. Here, analytes are separated as a function of molecular shape in addition to the mass-to-charge separation in the MS. The drift time is dependent on charge, size, shape, polarity, and collision cross-section and can provide isomer separation. Kylli et al. (2017) showed that the native oxysterol isomers did not separate well in ion mobility, but after derivatization with *p*-toluenesulfonyl isocyanate, separation was achieved for some of the oxysterol isomers (direct infusion to the MS, no chromatography in front). The derivatization also greatly enhanced the oxysterol signal in ESI, allowing for better detection limits. The combination of ultra-high pressure LC (UHPLC) and ion mobility MS allowed quantification down to 1 ng/mL from fibroblast cells. Overall, the results from Qiu et al. (2021) and Kylli et al. (2017) show the possibility of performing untargeted analysis of oxysterols using derivatization, LC, ion mobility, and MS for secure identification of new and known oxysterols.

MS can be operated on with several degrees of specificity. An untargeted analysis is to record compounds without preset filters. Targeted analysis implies a pre-set filtration, that is, a finite number of masses that are to be measured. The targeted approach can often lead to increased sensitivity and specificity, while untargeted “sterolomics” allows for a more versatile

approach and can be used to scout for novel oxysterols.

4.3 Sample Cleanup Before LC–MS Analysis

Different biosamples require various sample preparation techniques to extract the oxysterols and prepare the analytes for LC–MS analysis. After extraction (and possible derivatization), a sample cleanup is needed before chromatography, either to remove components from the sample that can interfere with the chromatographic separation and/or detection (matrix effects that can lead to ion enhancement or ion suppression, which can affect sensitivity and/or quantification) or to avoid undissolved material (e.g., cell debris or protein precipitate) that can clog the LC column, ESI unit, or even the mass spectrometer. Salts that have no retention when using RPLC will not interfere with the hydrophobic oxysterols but should not be introduced into the MS as salt contamination will increase the need for maintenance (e.g., cleaning of the MS inlet and lenses) and should therefore also be removed.

Solid-phase extraction is a selective sample preparation method that is used before LC. The sample is applied to a small cartridge, containing a stationary phase of choice, for example, a reversed-phase material. Non-retained compounds (e.g., salts, derivatization reagents) can be washed out using a non-eluting solvent. The oxysterols can then be eluted from the column using a stronger mobile phase, while more retained components (e.g., cholesterol or more hydrophobic materials) are retained in the column. Such one-time-use solid-phase extraction columns are widely used in bioanalysis and are available as single cartridges or in 96-well format.

The removal of cholesterol using these types of columns is effective, but to avoid autoxidation, cholesterol should be removed at an early stage (e.g., before derivatization steps), often resulting in the need to perform two different solid-phase extraction steps: one before and one after derivatization. Solid-phase extraction for the removal of excess derivatization reagent can also be

performed online by the LC instrument by using multitudes-use small LC columns.

4.4 Sample Preparation of Various Biosamples for LC–MS Analysis

4.4.1 Plasma and Serum

There are numerous examples of the determination of oxysterols in plasma and serum samples. To avoid the oxidation of cholesterol into oxysterols (autoxidation), which creates sample preparation artifacts and compromises the quantification, several careful steps should be included. The general procedure (see Fig. 4.2). seems to be to collect the blood sample on ethylenediaminetetraacetic acid (EDTA)-containing vacutainers, and plasma or serum is prepared by centrifugation. EDTA chelates Fe^{2+} and prevents the formation of free radicals and possible oxidation. The next step usually includes the addition of an antioxidant, such as butylated hydroxytoluene (BHT). The sterols are then extracted from the serum or plasma using either an alcohol such as ethanol or 2-propanol or versions of traditional lipid extraction methods such as the Folch method (2:1 v/v chloroform/methanol) or the Bligh and Dyer method (1:2 v/v chloroform/methanol). An additional effect of extraction with organic solvents is the precipitation of proteins. After collecting the resulting supernatant, cholesterol can be removed from the oxysterols, or autoxidation can be monitored by adding heavy isotope-labeled cholesterol to the sample: If autoxidation occurs, heavy isotope-labeled cholesterol will create heavy isotope-labeled oxysterols that can be monitored in the MS, and the sample can be discarded. Internal standards for quantification should also be added to the sample at an early stage, preferably before the removal of cholesterol.

Depending on the choice of analyzing total (nonesterified and esterified) or free (nonesterified) oxysterols, the sample preparation can include an alkaline hydrolysis step. Oxysterols are then (usually) derivatized into more

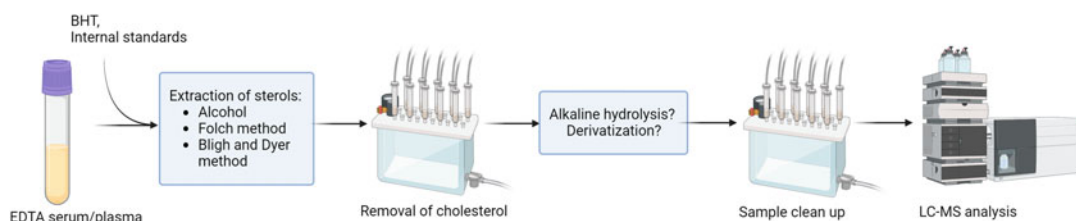


Fig. 4.2 Workflow for preparation of serum or plasma samples for oxysterol analysis. The sample is collected on ethylenediaminetetraacetic acid-containing vacutainers, and serum or plasma is created by centrifugation. Butylated hydroxytoluene is added to avoid oxidation, together with isotope-labeled internal standards. Oxysterols are extracted using a solvent (e.g., alcohol), and cholesterol

can be removed. If total oxysterols are to be determined, alkaline hydrolysis is included, often followed by derivatization to enhance MS sensitivity. Excess reagents and components that can compromise chromatography are removed during sample cleanup before LC–MS analysis for detection and quantification. Created with BioRender

MS-friendly derivates, followed by a sample cleanup and LC–MS analysis.

4.4.1.1 Analysis Without Derivatization

The comprehensive method developed by McDonald et al., with sterol determination without derivatization, has been attempted to be adopted by others (Roberg-Larsen et al. 2015; Mendiara et al. 2018), but as the author states, the formation of ammonium adducts for enhanced detection is highly instrument (or, more precisely, ionization source) dependent. Nevertheless, the direct analysis of oxysterols using LC–ESI–MS after protein precipitation and sample cleanup is possible. The MS signal is usually detected with the loss of one or two water molecules ($[M + H - H_2O]^+$) (Roberg-Larsen et al. 2015; Mendiara et al. 2018; Dias et al. 2018a).

The approach can be used to determine both free (nonesterified) and total (nonesterified and free) oxysterols. Mendiara et al. (2018) used the enzyme *cholesterol esterase* to avoid alkaline hydrolysis, resulting in improved recovery and less background noise as the enzyme is more specific and avoids the release of triglycerides and fatty acids that can interfere with the chromatography. Overall, the utilization of enzymes for hydrolysis reduces the need for sample cleanup and elevated temperatures during sample preparation and is an approach that should be further investigated and adapted for total (esterified and nonesterified) oxysterol determination.

Helmschrodt et al. (2013) use an alternative ionization source to ESI, APCI, to determine oxysterols in plasma after fast and simple sample preparation. APCI is less dependent on the analytes' charge-readability and is arguably a suitable component in the analysis of underivatized oxysterols. Proteins were precipitated and sterols were extracted using methanol and 2-propanol, followed by evaporation of the solvent, re-desolvation, and centrifugation before the analysis. The method was applied for the determination of free 7-keto, 7 α / β -hydroxy, 5,6 α -epoxy, 5,6- β -epoxycholesterol, cholestane-3 β -,5 α ,6 β -triol and cholesterol in both human plasma and atherosclerotic plaques, which are all linked to the initiation and progression of atherosclerosis. The authors used isotope-labeled cholesterol for autoxidation monitoring, showing no autoxidation. The isomers were separated on a monolithic column (Chromolith SpeedROD RO-18e), which is a one-piece polymer synthesized within the column, typically associated with a lower back pressure compared to conventional LC and UHPLC, an LC variant with smaller particles (high back pressure but can allow for more rapid separations). The method's robustness with this simple sample preparation was not discussed. The method has been further developed to determine nonesterified cholesterol, 17 nonesterified oxysterols, and 17 free and conjugated bile acids in plasma and cerebrospinal fluid (Reinicke et al.

2018). The extended method also contains an additional sample cleanup step using an automated online solid-phase extraction column switching system, which allows large injection volumes (200 μ L) to enhance detection limits. The method was applied to study patients with blood–brain barrier disturbances, but no significant differences were detected compared to controls. The method has also been adopted by Kloudova-Spaenkova et al. to study the circulating levels of seven free oxysterols during the progression of luminal subtype breast cancer. They found that the circulating levels of 7- α -hydroxycholesterol, 26-hydroxycholesterol (also referred to as 27-hydroxycholesterol), cholesterol-5 β ,6 β -epoxide, and cholestan-3 β ,5 α ,6- β -triol were lower in patients with small tumors compared to large and later-stage cancers.

4.4.1.2 Analysis with EADSA Derivatization

Griffiths established that EADSA is a popular choice for analyzing oxysterols and is applied in plasma and serum samples as well (Griffiths et al. 2013). The approach enjoys excellent sensitivity and characteristic MS/MS spectra, aiding the detection, identification, and elucidation of sterols. This method has been adapted by others and can be used with both Girard P and T reagents. A drawback with this method is the creation of syn and anti forms during the derivatization, resulting in double peaks on traditional C18 stationary phases. This seems not to be an issue for hydroxycholesterols when a phenyl-based reversed-phase stationary phase is applied. In addition, the method does not separate oxysterols with naturally occurring ketone groups at position 3 from those with a hydroxyl group. Hence, samples should be divided into two, prepared with and without the enzyme cholesterol oxidase, and analyzed separately. The development of different derivatization tags for EADSA allows multiplexing, reducing the analysis time on the LC–MS. Nevertheless, the need for two sample preparations on the same sample complicates the analysis. The method has been used extensively in the group of Griffiths and Wang but has also been adapted by others

(DeBarber et al. 2010, 2011; Roberg-Larsen et al. 2014, 2017; Soroosh et al. 2014; Solheim et al. 2019).

4.4.1.3 Other Derivatization Approaches

The derivatization of oxysterols into picolinyl esters (a method established by Honda et al. 2008) is suitable for the determination of oxysterols in small-volume plasma samples. Xu et al. (2013) modified the method to determine the total (esterified and nonesterified) concentration of 4 β -hydroxycholesterol and cholesterol in 5 μ L of human and mouse plasma. The ratio between 4 β -hydroxycholesterol and cholesterol was suggested as an endogenous biomarker for CYP3A4/5 activity. After alkaline hydrolysis, the oxysterols were derivatized into picolinyl esters, followed by a liquid–liquid extraction step using hexane for sample cleanup. In contrast to Honda et al., Xu et al. created sodium adducts in their MS, emphasizing the importance of MS method optimization. The 4 β -hydroxycholesterol was also derivatized in two positions (3 and 4), while the Honda approach resulted in the addition of one group. The addition of one or two groups should be optimized, as these derivatives will give different retention times in reversed-phase chromatography and can make the chromatographic separation challenging (Nadarajah et al. 2017) or even lead to false quantifications.

Marelli et al. have also adopted the Honda method for the determination of oxysterol related to spastic paraplegia type 5. Aliquots of 20 μ L of EDTA plasma were added to internal standards and BHT in methanol, followed by alkaline hydrolysis and derivatization into picolinyl esters. Interestingly, the ESI was set to negative mode, usually most suited for anions and not cations, which the derivatized oxysterols were in this approach. The authors, however, do not state if they monitored sodium adducts or the precursor ions or fragments monitored in the MS/MS analysis. Nevertheless, Marelli et al. could conclude that 25-hydroxycholesterol and 26-hydroxycholesterol and their ratio to total cholesterol could discriminate between spastic

paraplegia type 5 patients and healthy controls with 100% sensitivity and specificity.

N,N-dimethylglycine derivatives of oxysterols have been shown to have good chromatographic traits. Pataj et al. (2016) separated the isomers on a biphenyl column using a fast gradient, resulting in baseline separation of the isomers in 7.8 min. The method was applied to human plasma and red blood cells with the aim of doing an extensive investigation into rare diseases such as Niemann-Pick type C, cerebrotendinous xanthomatosis, and neurodegenerative or cardiovascular diseases.

4.4.2 Cultured Cells

For measuring cell content, LC-MS of oxysterols is fully applicable in several formats. For example, conventional columns (>1 mm ID) can be used for ample samples, or more down-scaled variants can be employed for limited samples (Roberg-Larsen et al. 2012). For example, Lundanes and co-workers applied nano-LC columns for the analysis of oxysterols in cancer cell samples. The nano-LC system allowed for measurements at the thousand-cell scale (Roberg-Larsen et al. 2014) (Fig. 4.3). However, there is currently a major push toward single-cell analysis. Although fairly conventional preparation methods were applied in Roberg-Larsen et al. (2014), that is, ethanol-based extraction from cells, in-vial derivatization, etc., a single-cell analysis will undoubtedly require dramatic miniaturization of the sample preparation with precise robotic systems for ultra-small samples, including lysis, derivatization, and sample cleanup, as seen in, for example, single-cell proteomics (Røberg-Larsen et al. 2021).

4.4.3 Tissue

4.4.3.1 Brain Tissue

Oxysterols in both human and mouse brains have been studied with different approaches in relation to both Alzheimer's disease and development. Dias et al. (2022) studied oxysterols and oxysterol

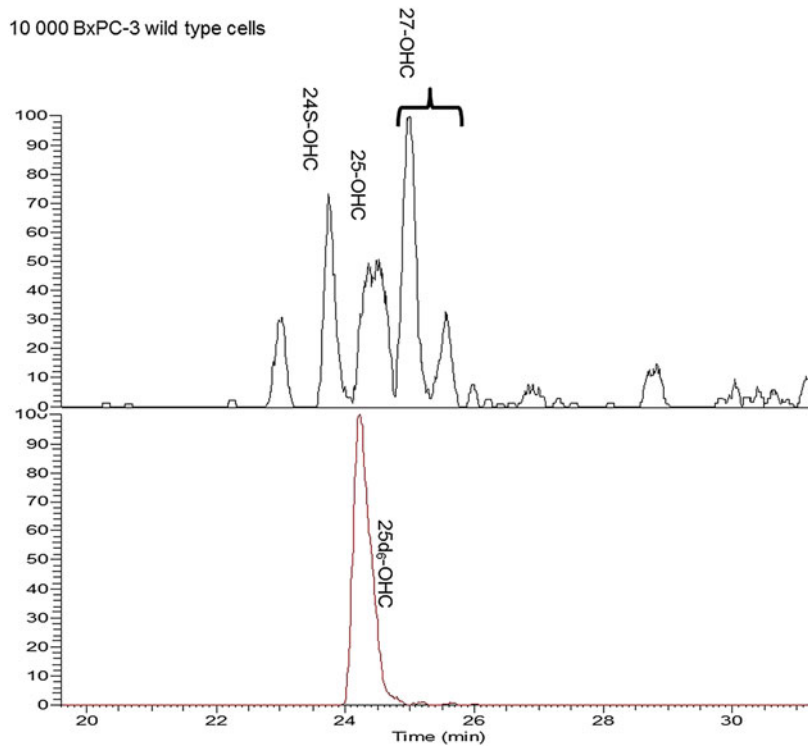
sulfates in Alzheimer's disease brains after homogenization and extraction with methanol and BHT. Sample cleanup and enrichment of oxysterols were performed on an offline polymeric SPE column, followed by LC-MS analysis without derivatization. With the developed method, they observed that enzymatically generated 26-hydroxycholesterol and nonenzymatically generated 7-oxysterol were significantly elevated in Alzheimer's disease brain tissue. Ahonen et al. (2014) studied oxysterols and vitamin D metabolites in mouse brains using APPI for enhanced detection. Meljon et al. (2012) use EADSA to analyze oxysterols in newborn mouse brains. Oxysterols were extracted from the brain using methanol:chloroform (1 + 1), followed by removal of cholesterol and derivatization with Girard P reagent. The method was used to map bioactive oxysterols in a newborn mouse brain, identifying several oxysterols derived from demisterol and high levels of 24S,25-epoxycholesterol, a potent LXR and Insig ligand.

MS imaging (MSI) has also been used to map cholesterol in the brain after on-tissue derivatization following the EADSA method and matrix-assisted laser desorption ionization or desorption electrospray ionization (Angelini et al. 2021). The method showed how the cholesterol distribution changes during development in mouse brains using isotope-labeled standards for quantification. This approach has also been applied to oxysterols and sterols (Yutuc et al. 2020); however, the oxysterol isomers require separation before MS detection, and microscale liquid extraction for surface analysis (μ LESA) was applied to extract spots with 400 μ m spatial resolution and transfer them to the LC-MS for separation, identification, and detection (Fig. 4.4).

4.4.3.2 Breast Cancer Tissue

26-Hydroxycholesterol (also referred to as 27-hydroxycholesterol) has been linked to breast cancer (Wu et al. 2013; Nelson et al. 2013), and this has been studied in tumor tissue, both ER-positive (ER+) and negative (ER-). Wu et al. (2013), using the McDonald method, showed that 26-hydroxycholesterol increased in

Fig. 4.3 Chromatogram of side-chained hydroxycholesterols extracted from cultured pancreatic adenocarcinoma cells using nano-LC-MS. Analytes were extracted from the cells using ethanol and Girard T-derivatization. Reprinted with a CC-BY license from Roberg-Larsen et al. (2014)



human ER+ breast cancer tissue compared to normal breast tissue. Solheim et al. (2019) used the EADSA method with Girard T and online sample cleanup in combination with fast LC-MS to examine both free and total oxysterol concentrations in breast cancer tissue from both ER+ and ER- tumors. The study revealed large intratumor variations in the concentration of different enzymatically formed oxysterols. The variation was observed both in absolute and relative concentration, implying that the extraction efficiency of sterols from the tissue was not the issue. The findings linking 26-hydroxycholesterol with breast cancer are also in contrast to the circulation of 26-hydroxycholesterol in the blood, where a large study (involving 530 individual patients) revealed that higher circulation of 26-hydroxycholesterol was associated with a lower risk of breast cancer in postmenopausal women (Lu et al. 2019). This finding highlights the importance of studying smaller sample selections, for example, tissue or exosomes (see below), to understand the role of oxysterols in, for example, breast cancer.

4.4.4 Exosomes

Exosomes are small vesicles that are produced by cells and released into the extracellular space. They are involved in intercellular communication and play a role in various physiological and pathological processes. Cancer cells may also release exosomes with cargo that can promote further disease development. Exosomes are typically 30–150 nm in size and contain a variety of biomolecules, including proteins, and nucleic acids. In addition, exosomes may feature small molecules such as lipids. R berg-Larsen and co-workers applied LC-MS methodology with online solid-phase extraction for measuring oxysterols in exosomes from cell lines, including breast cancer cell lines MCF-7 (ER+) and MDA-MB-231 (ER-). While side-chain hydroxylated oxysterols did not have outstanding levels in the cells compared to healthy cells, the level of 26-hydroxycholesterol was significantly higher in MCF-7 exosomes (Fig. 4.5). This result could be interesting regarding disease

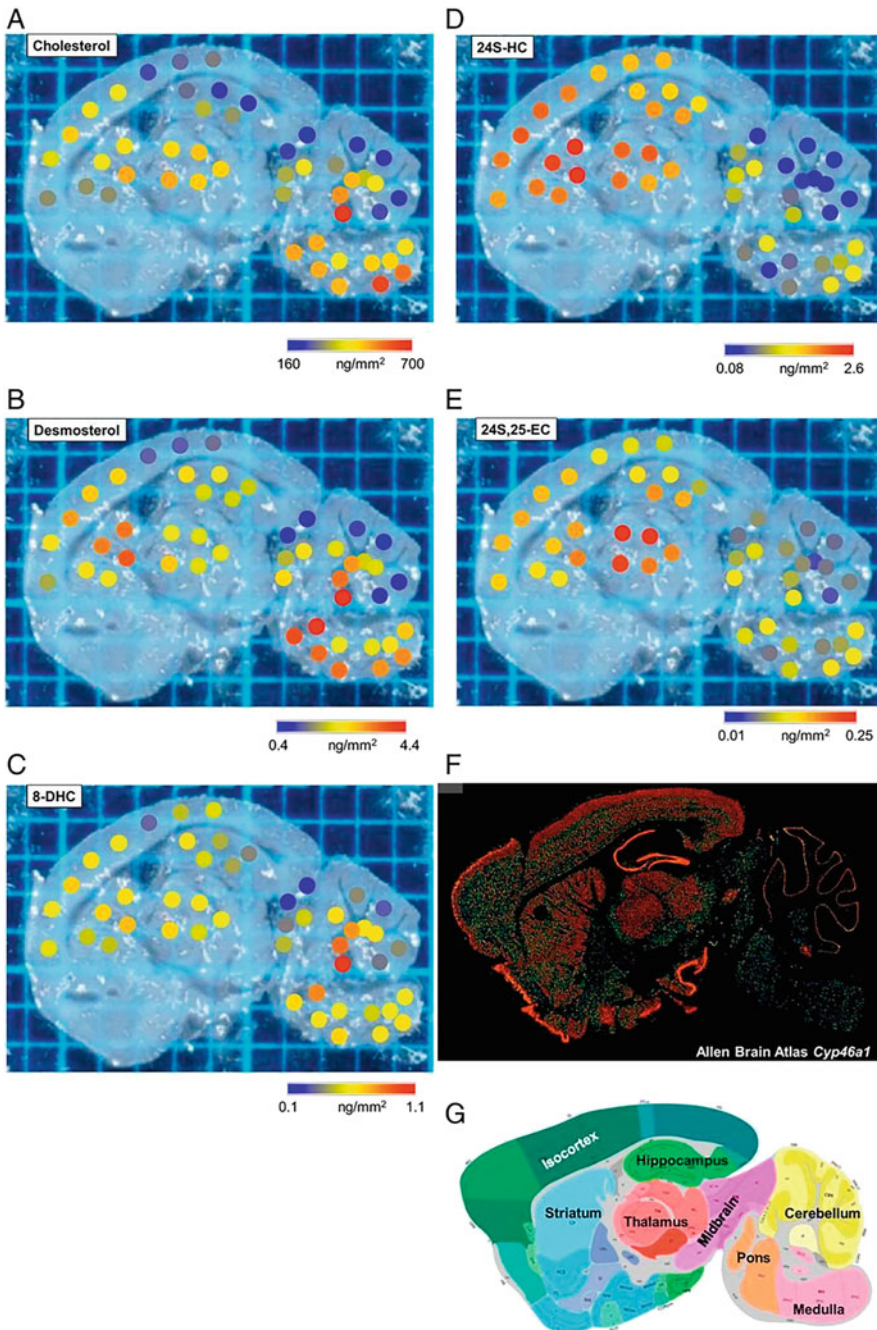


Fig. 4.4 Mouse brains were analyzed using μ LESA-LC-MS after EADSA for sterol detection and isotope-labeled quantification. Reprinted with a CC-BY license from Yutuc et al. (2020)

development, as 26-hydroxycholesterol has been shown to be associated with cancer proliferation in ER+ cancers (Wu et al. 2013; Nelson et al.

2013). A key challenge in exosome analysis is their availability; extraction from biosamples will only result in limited amounts (e.g., <1 μ g

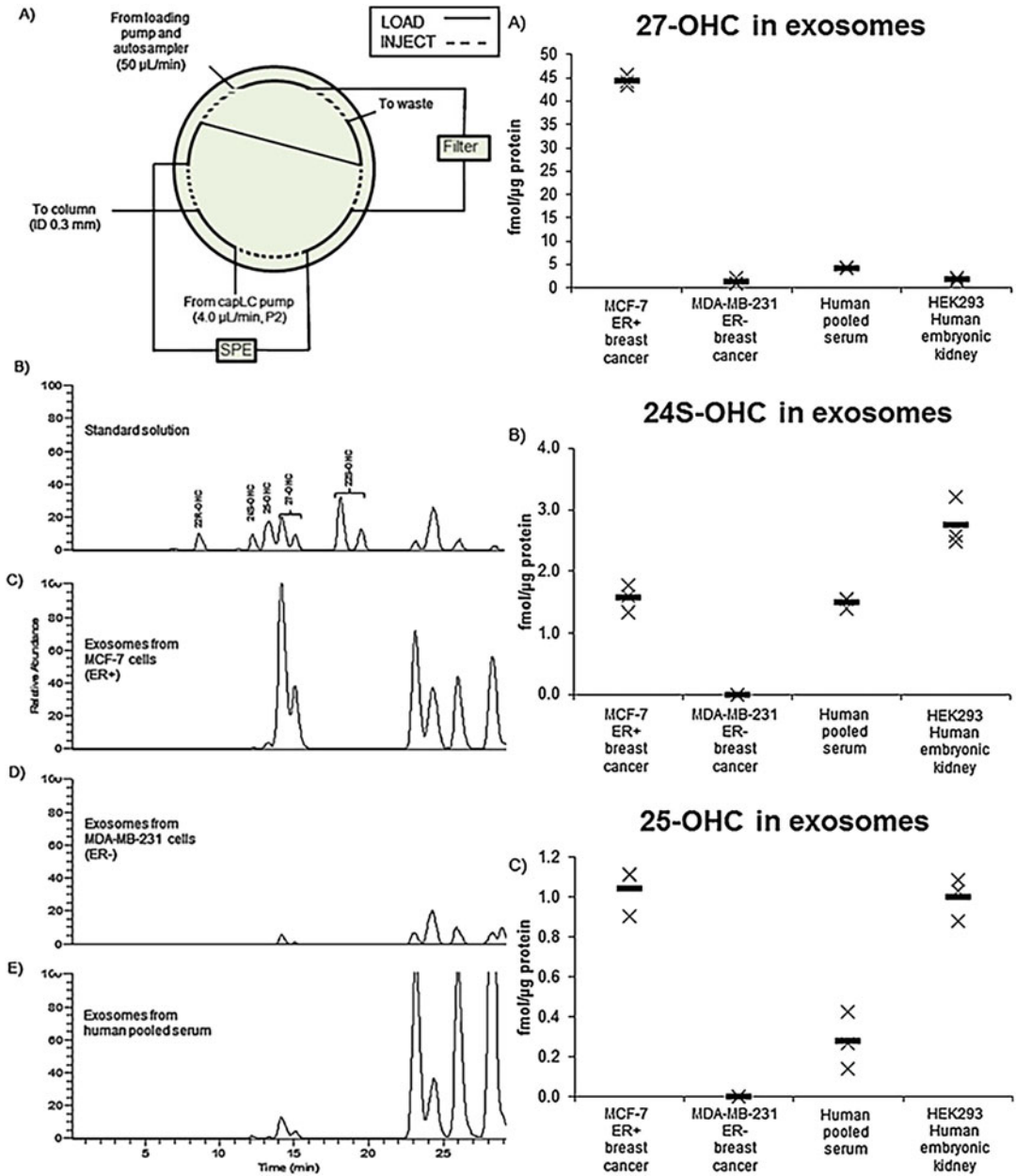


Fig. 4.5 Upper left: an overview of the automatic filtration and filter back flushing-solid-phase extraction-liquid chromatography system plumbing applied for the sample preparation and analysis of oxysterols in exosomes. Lower left: representative chromatograms of oxysterol analytes from exosomes of cancer cells and exosomes

from human serum. Right: levels of oxysterols revealed a significant increase of 26-hydroxycholesterol (also referred to as 27-hydroxycholesterol) in exosomes from estrogen receptor-positive cells, compared to similar oxysterols. Reprinted with permission from Roberg-Larsen et al. (2017)

exosome protein/million cells). Therefore, it is imperative to have access to highly sensitive

analytical platforms. For the study described here, capillary LC was employed (a downscaled

variant of conventional LC using sub 1 mm inner diameter columns). For lysing and extraction of oxysterols, the same sample preparation approach used for nano-LC–MS of cells was applied, showing that the simple ethanol extraction-based protocol can be used for several kinds of related samples. Perhaps, a greater challenge with exosomes is securing the contamination-free isolation of these vesicles; the exosomes analyzed in this study (obtained commercially) had been isolated using a polymer precipitation approach. Exosomes were characterized by Western blot and NanoSight for size and intactness. Size distribution analysis did not reveal a significant presence of lipoproteins.

4.4.5 Organoids

Organoids are laboratory-grown organ models and are emerging tools for developmental biology, drug discovery, and disease modeling, to mention a few of their many applications. Organoids can be grown from, for example, induced pluripotent stem cells into miniature models of, for example, the liver, kidney, and even brain. Liver organoids can, for example, be used to study nonalcoholic fatty liver disease by subjecting liver organoids to excessive amounts of lipids, resulting in the liver organoids developing various stages of the disease (see Fig. 4.6). A key challenge with organoids can be that a single organoid is <1 mm in size, which can present issues with sensitivity, as with other limited sample amounts and sizes. Organoids have not been widely explored regarding oxysterols. However, an early study by K murcu et al. showed that oxysterols, both dihydroxysterols and hydroxycholesterols, were found in elevated levels in the medium of treated organoids compared to controls, implying that the organoids secrete oxysterols as a response to infection (K murcu et al. 2023). Approximately 90 μL of the organoid medium was mixed with internal standard cholesterol-25,26,27- ^{13}C for autooxidation monitoring. Samples were evaporated to dryness and redissolved in 20 μL

2-propanol before Girard T-derivatization. These results were also in accordance with a few other studies of NAFLD using blood from patients, suggesting that oxysterols may have a potential for serving as biomarkers of the disease and calling for further studies. The sample preparation was similar to that of tissue samples (Solheim et al. 2019) and also featured automatic filtration and filter flush-solid-phase extraction-LC–MS for the sample cleanup, separation, and detection of the analytes. However, the system was not capable of quantifying the low levels in the control samples, and additional oxysterols may be present in the liver organoids with diagnostic value. Therefore, the sample preparation may need to be downscaled for further gains in sensitivity.

4.5 Conclusion

The preparation and analysis of oxysterols have been explored, focusing on approaches centered around LC and MS. Oxysterols required substantial attention in every step, including: addressing the possibility of autooxidation during sample preparation, which can perturb accurate quantification; considering a derivatization step, which may enhance sensitivity and mass spectra quality, but may require several additional steps in the procedure; how to remove potentially interfering compounds and materials, for example, through the use of solid-phase extraction, either in an online or offline manner; how to separate the oxysterols in a selective fashion, to not mix structurally similar but biologically distinct isomers; addressing potential adducts that may form due to the addition or absence of sample preparation steps. Finally, these steps must be applied and modified for a range of biomaterials, from ample amounts of blood samples to potentially limited samples such as exosomes.

However, oxysterol sample preparation has been vigorously explored and optimized in several directions. Today, we can measure oxysterols in a highly sensitive and selective manner, unraveling biological functions and biomarkers. In this regard, one can say that the field is a

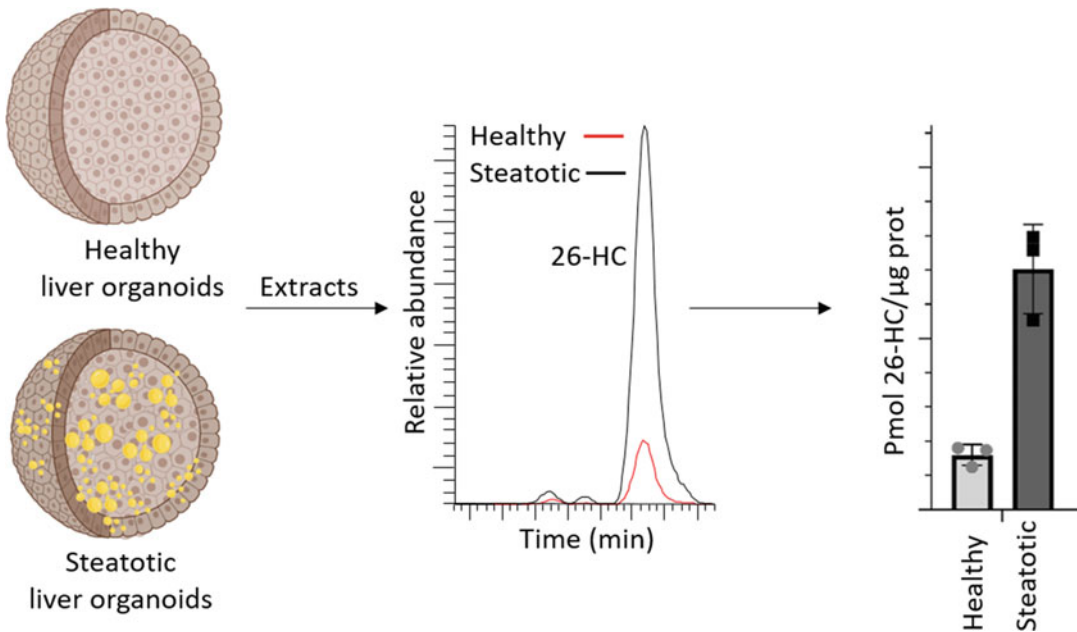


Fig. 4.6 Steatosis was induced in liver organoids, followed by measurements with LC–MS, which revealed that 26-hydroxycholesterol (also referred to as 27-hydroxycholesterol) was significantly upregulated in

the secretion of treated organoids. As these samples are quite limited, efforts are being made to downscale the sample preparation. Reprinted from K murcu et al. (2023)

success. Still, improvements regarding ease of preparation and throughput would be very welcome as we continue to seek new oxysterol variants in complex and minute samples, potentially of clinical interest and for the health of all of us.

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