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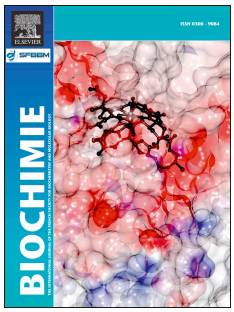

CHROMATOGRAPHY OF OXYSTEROLS

Irundika H.K. Dias, Steven R. Wilson, Hanne Roberg-Larsen

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Abstract

Oxysterols play important roles in development and diseases, but can be highly challenging to analyze. To ensure satisfactory measurements, oxysterols must typically be separated with chromatography prior to detection. Here, we will devote attention to the chromatography of oxysterols, focusing on gas chromatography and liquid chromatography. We will present the role of stationary phases, mobile phases, and dimensions and geometries of particles/columns. We discuss how these parameters may affect the chromatography, regarding factors such as speed and resolution. Finally, we present some less explored avenues for separation of oxysterols.

CHROMATOGRAPHY OF OXYSTEROLS

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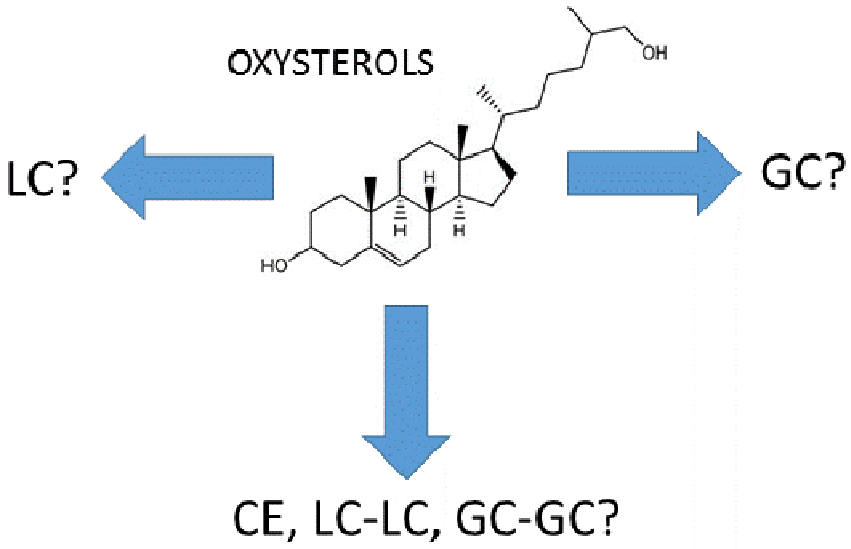

Highlights

- Gas chromatography for oxysterols is well established
- More diversity in liquid chromatography approaches
- Approaches to enhancing speed and selectivity with LC are presented
- Few differences in chromatography between native and derivatized oxysterols
- Alternative approaches to separation of oxysterols exist, but are little explored

Key words (max 6)

Oxysterols; Chromatography; Separation; Lipids; Sterols

Abstract

Oxysterols play important roles in development and diseases, but can be highly challenging to analyze. To ensure satisfactory measurements, oxysterols must typically be separated with chromatography prior to detection. Here, we will devote attention to the chromatography of oxysterols, focusing on gas chromatography and liquid chromatography. We will present the role of stationary phases, mobile phases, and dimensions and geometries of particles/columns. We discuss how these parameters may affect the chromatography, regarding factors such as speed and resolution. Finally, we present some less explored avenues for separation of oxysterols.

1. Introduction

Oxysterols are a group of lipids that receive considerable attention due to the unraveling of their roles in numerous diseases and development [1-5], and are established biomarkers for e.g. Niemann–Pick disease (NPD) [6]. Quality measuring tools must be employed to understand the roles oxysterols play in development, diseases and conditions. However, the measurement of oxysterols can be highly challenging. Some reasons are that oxysterols may be present at low concentrations, in limited samples. In general, such issues can often be solved by using highly sensitive mass spectrometry (MS) techniques. However, many oxysterols are not "ideal" for MS analysis, as they can be difficult to ionize; ionization is key requisite when using electrospray ionization (ESI), a most common interface of MS. But perhaps equally important, oxysterols are often highly similar compounds, e.g. present as isomers with similar MS fragmentation profiles, making selective determinations a significant challenge. Thus, oxysterols require particular care regarding pre-MS steps. A key step is to ensure high quality chromatographic separations, for e.g. resolving isomers and achieving precise measurements.

In this review, we will focus on the chromatography of oxysterols. In particular, we will discuss separations of oxysterols using gas chromatography (GC) and liquid chromatography (LC), giving attention to the speed, resolution and sensitivity of oxysterol separations using these techniques. Although we acknowledge the great improvements made in separation instrumentation over the years (rapid injection systems, low void volume connections, MS resolution etc.), we focus here on fundamental separation conditions, e.g. column materials, stationary phases, mobile phases (MP) and particle geometries. The chromatography of native and derivatized oxysterols ("charge-tagged" for improved MS sensitivity) will be discussed. Finally, we will take a look at some less employed approaches for separations, which may have future roles in oxysterol separations.

2. Gas Chromatography and oxysterols: "Never change a winning team"?

GC is a technique in which compounds are separated in meter-scale columns with inner diameters well below 1 mm. Compounds are separated by having unequal retention factors (time spent on the column walls/ time spent in a gaseous MP). The stationary phase is typically a polymer coating around 0.25 µm in thickness. GC can provide excellent resolution and is simply coupled with MS, typically via election ionization (EI) interfaces. Also, GC-EI-MS does not suffer from suppression effects to the same degree as ESI (the common MS

interface with LC) [8, 9]. Reduced suppression from other compounds lessens the need for analyte-specific internal standards. GC has been a workhorse for analysis of sterols for well over 50 years [10-12]. For the last couple of decades, a method described by Ulf Diczfalusy and co-workers for oxysterol analysis has been highly influential [13]. For measurements of the analytes in human plasma, the authors separated 7α- and 7β-hydroxycholesterol, 7-oxocholesterol, cholesterol-5α,6α-epoxide, cholesterol-5β,6β-epoxide, cholestane-3β,5α,6β-triol, 24-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol and used MS for detection (**Figure 1**). The analytes were derivatized with trimethylsilyl (TMS) to enhance volatility as required by GC. The authors employed a 30 meter long column, featuring a HP-5MSM ((5%-phenyl)-methylpolysiloxane) film, which is described as a non polar "general purpose" stationary phase, where separations are mainly based on differences in boiling points. A temperature gradient was employed and the MP (helium) was held at 0.8 mL/min (35 cm/second), which is close to the optimal column efficiency for the MP and column inner diameter (ID) (0.25 mm). Using these conditions, the authors obtained peaks with widths of about 15 seconds, with all the analytes being detected within 19 minutes.

This method has been cited several hundred times, and more importanty, has been reproduced/re-used in a substantial number of studies. Quite remarkably, few major significant modifications to this GC method have been reported. Some researchers have reduced the analysis time; for determining serum cholestane-3β,5α,6β-triol as a biomarker for Niemann-Pick type C disease (NPC) diagnosis, Kannenberg et al. performed analyses below 10 minutes per sample. The authors employed a trifluoropropylmethyl polysiloxane phase, a mid-polar stationary phase, which is promoted as being ideal for separating positional isomers [14]. A most notable reduction of analysis time was shown by Maria T. Rodriguez-Estrada and co-workers, who performed analysis of cholesterol oxidation products using "fast gas chromatography" [15]; a shorter (10 m) column with a narrower ID (0.1 mm) and thinner stationary phase film (0.1 µm thickness) allowed for separation (resolution (Rs)>1.2) of 7α-hydroxycholesterol, 19-hydroxycholesterol, 7β-hydroxycholesterol, β-epoxycholesterol, α-epoxycholesterol, cholestanetriol, 25-hydroxycholesterol, 7-KC in 3.5 minutes (**Figure 2**). It should be noted that a narrow ID and thin film allow for improved efficiency, which can be essential for obtaining resolution when the column length is decreased (reduces analysis time, but affects the separation). The fast analysis time of this method is roughly 5-10 times faster than that described in most related papers. The flow rate was 0.41 mL/minute, which corresponds to a linear velocity of 43 cm/second (narrow columns can be operated at higher linear velocities without dramatic decreases in efficiency). However, substantial efforts

126 towards gas chromatographic improvement have in general not been prioritized in oxysterol
127 analysis. This is perhaps quite reasonable, as GC has to a large degree reached a level of
128 maturity allowing for stable analysis, especially when analysis time is not a major concern.
129 Also, GC-MS instruments are typically less expensive than LC-MS instruments.
130
131 **3. Liquid chromatography and oxysterols: a broad range of approaches**
132 Briefly, LC is a technique in which compounds are separated in a centimeter-scale column
133 with an inner diameter typically between 1-4 mm. The MPs are liquids, and the stationary
134 phases are often attached to particles with sizes typically between 1.7 and 5 µm diameters.
135 Although GC can provide greater plate numbers per column, LC provides greater plate
136 numbers per column length. Moreover, derivatization is not an absolute requirement for all
137 applications. Regarding oxysterols, LC-ESI-MS is generally more sensitive than GC-MS
138 variants [16]. Oxysterol separations by LC have been undertaken using either normal-phase
139 (NP) LC or reversed-phase (RP) LC.
140
141 *3.1 NPLC: a lost cause?*
142 In NPLC, molecules are adsorbed to the surfaces of silica particles (unmodified or featuring
143 polar chemically bonded phases) and are eluted with non-polar solvents such as hexane or
144 heptane. Analytes engage with the stationary phase via hydrogen bonding and dipole
145 interactions. There are some examples of determination of oxysterols using NPLC coupled to
146 UV detection with good chromatographic resolution between the isomers [17, 18]. However,
147 NP MP solvents show low conductivity, surface tension, and lack ability to donate or accept a
148 proton to give analytes charge (and hence sensitivity) for ESI-MS detection (a “gold-
149 standard” in the analysis of fluids). On the other hand, this can be partially overcome by
150 addition of polar solvents such as 2-propanol [13] or methanol [14]. But in addition, NP is
151 generally perceived as having lower reproducibility and predictability than RPLC. Normal
152 phase and reverse phase separations have been compared regarding oxysterols. Careri et al.
153 compared chromatographic separations of cholesterol and five oxysterols using NPLC
154 (Nucleosil 5-CN column) and RPLC (C18, Ultracarb ODS (20) column). RPLC (associated
155 with more robustness) provided superior sensitivity for all the compounds measured [19].
156 RPLC is the key mode when analyzing oxysterols in complex biological samples with MS
157 detection. However, we will discuss promising separation techniques related to NPLC in the
158 final section.
159

3.2 RPLC: a workhorse with possibilities for different selectivity

RP stationary phases are hydrophobic and are typically alkyl chains. The oxysterols, both native and derivatized are most commonly separated using octadecyl alkyl chain (C18)-bonded silica stationary phases, followed by C8-bonded silica columns (**Tables 1 and 2**). The analytes are expected to elute according to hydrophobicity (although RPLC is far more complex than commonly perceived [20]). With RPLC, the more polar side chain oxygenated oxysterols elute before ring-oxidized sterols followed by more non-polar sterols [19, 21]). C18 columns generally provide the same retention order for oxysterols (when using similar MPs) regardless of the derivatization reagent (derivatization in LC is discussed in some more detail below). This implies that selectivity differences are typically not attributed to the derivatization reagent. However, RPLC can have somewhat surprisingly degrees of selectivity options. Shan et al. compared oxysterol separations regarding two solvent systems, acetonitrile:water and methanol:water, on C8 and C18 columns [22]. They showed that even with same MP the chromatographic mobility and selectivity between C8 and C18 columns are considerably different. The C8 column was able to resolve several oxysterol pairs, including 7α- and 7β-hydroxycholesterol, which were inseparable on the C18 column using a similar MP. Compared to using methanol:water, using acetonitrile:water with a C18 column provided improved resolution of oxysterol pairs 27-hydroxycholesterol/3β,5α,6β-triol, 24R-hydroxycholesterol/20α-hydroxycholesterol, 7α-hydroxycholesterol/7β-hydroxycholesterol, and 7-ketocholesterol/3β-OH-6-one. Roberg-Larsen et al. observed that employing an acetonitrile-based MP and C18 allowed separation of 20-hydroxycholesterol and 27-hydroxycholesterol, while separation of 27-hydroxycholesterol and 24S-hydroxycholesterol was not possible with the same conditions [23]. The opposite was observed when using methanol-based MP (in the final section we will discuss an approach that may allow these two systems to be combined). Roberg-Larsen et al. has observed highly similar RPLC oxysterol separations in microbore LC, capillary LC and nano LC [23-25] (selectivity is rarely affected by column diameter).

24S-hydroxycholesterol and 25-hydroxycholesterol can also be challenging to separate. Debarber et al. separated 24R- and 24S-hydroxycholesterol by modifying a method by Burkard and coworkers [26] using a methanol:acetonitrile:water MP (45:40:35) and a column temperature of 55 $^{\circ}$C [27]. Changing to methanol/acetonitrile/water (14:0.6:1) and a column temperature of 10 $^{\circ}$C same authors demonstrated separation of 24-hydroxycholesterol from 25-hydroxycholesterol within short (6.5 min) time. The later method reversed the retention

order of two oxysterols to 24-hydroxycholesterol followed by 25-hydroxycholesterol. However, the later method did not baseline separate the two oxysterols.

Great efforts have been made to ensure that the analytes do not interact with the particles in which the stationary phase is attached, as e.g. silica particles cause secondary interactions and may perturb the separation (e.g. cause band broadening and tailing). Avoiding interactions with the particles can be done by efficient rest-silanization and adding functional groups at the trunk of the main stationary phase for steric hindrance. In addition, the carbon loading of the particles is crucial for oxysterol separations. For instance, otherwise high quality columns which featured lower carbon loads were unable to provide selective separation of side-chained oxysterols [25].

To improve chromatographic efficiency further, the size of the particles may be reduced; today sub-µm particles are common, while 3-5 µm particles were standard about a decade ago. The Hypersil GOLD^{TM} column is a familiar column in oxysterol RPLC, both in UHPLC (sub 2 µm particles) and regular HPLC (3 µm particles), typically with a 2.1 mm ID. The Hypersil GOLD^{TM} columns are endcapped silica-based columns, with a high hydrophobicity and medium shape selectivity and polar surface activity. (For column classification see [28] and [29]) This column material has been used for chromatographing both Girard P- and Picolinyl ester derivatized (PED) oxysterols [30-39]. This column seems to not be compatible with Girard T derivates (Rs >1.1, data not published). However, sub-µm particles cause higher back-pressures. Therefore, a highly attractive alternative has been the use of core shell particles. Core shell particles have a solid core and porous shell, that gives high efficiency and fast separations with low back pressure compared to traditionally porous particles [40]. Core shell particles provide similar efficiencies to sub-µm particles. A well-known example of core shell particles for oxysterol analysis is that by McDonald et al. who chromatographed 62 different sterols, oxysterols and secosteroids from human plasma using two different LC and one GC method. Both the LC methods used core shell particles, with the side-chain oxysterols eluting in 7.5min (total run time 12min) [41].

A notable exception from using octyl chain stationary phases is by Silke Matysik and co-workers who employed a biphenyl phase [42]. Biphenyl stationary phases typically provide increased retention and can have a different selectivity compared to traditional C18/RP phases, as it can provide both π –π interactions and higher hydrogen bonding capacities [43]. In addition to featuring a different phase, the column employed was packed with core shell

particles. The work of Silke Matysik and co-workers demonstrate a quick separation of N,N-dimethylglycine (DMG) derivatized oxysterols (8 oxysterols in 8 minutes, see **Figure 3**)

3.3 Effect of derivatization on oxysterol chromatography

Derivatization of oxysterols is used to enhance sensitivity in MS detection by incorporation of a charge group into the oxysterol. Derivatization of oxysterols can be used with both ESI-MS and atmospheric pressure chemical ionization (APCI)-MS. In addition to enabling enhanced sensitivity, derivatization can also make the sterols more soluble in MPs commonly used in RPLC. Derivatization can also prevent adsorption of the hydrophobic sterols on narrow ID fused silica tubing in use in sensitive nano LC-based systems [7]. Other benefits of derivatization are more easily interpretable MS2 spectra, as fragmentation of the derivatized group usually gives more specific fragmentation [7].

A variety of different derivatization reactions for sterols exist, and most common used ones for oxysterols are Girard P and T reagent, picolinyl acid and DMG (for end product structures see **Figure 4** and a recent review by Yuqin Wang and William J. Griffiths' group summarizes the details for all the most common derivatization reactions [4]. While DMG is mostly used in the context of NPC disease [44-46], Girard P and T is used in the context of neurologic [31] or metabolomic [33] diseases and cancer [23, 24]. All these derivatization reactions are targeting the hydroxyl group. An alternative is to use click-chemistry, to target the double bond between the C5 and the C6 in the sterol structure, e.g. by thiol-ene click-chemistry tagging using a photoinitiator [47]. The click-chemistry generates heteroatom links (C-X-C) and reaction rates can be quick (< 1 minutes) when using a microflow reaction cell.

Regarding chromatographic performance, there are small differences in the behavior of the derivatized or native oxysterols. Cha et al. [48] has analyzed both native oxysterols from serum samples as silver adducts and picolinyl ester derivatized (PED) oxysterols from CFS. Although the chromatograms look very different regarding analysis time, the analysis is performed on two different reversed phase columns; An ACE C18 (3 µm, 150 mm x 2.1 mm ID) and a Kinetex C18 (2.6 µm, 100 mm x 2.1 mm ID, core shell). Although these columns has approximately the same hydrophobicity, they have different shape selectivities and polar surface activities [28] and most importantly, different solid supports (fully porous vs. core-shell). It would be interesting to compare the separation of the PED with McDonald et al. [41], and the native oxysterol separation with Roberg-Larsen et al [24], which both uses the

same columns on native and Girard T derivatized oxysterol, respectively. McDonald's oxysterol-ammonium adducts shows similar chromatography and elutes in the same retention window as Cha's PED, while Roberg-Larsen's Girard T derivates elutes in the same retention window as Cha's native sterol.

3.4 Dimensions and sensitivity

The most popular column dimension used in oxysterol analysis is the 2.1 mm ID format. Detection limits for native oxysterols and all the types of derivates are in low ng/mL, suitable for analyzing oxysterols in plasma. However, most of the applications use more than 50 µL plasma or serum in their sample preparation. The relatively high sample volume for the other methods can be challenging if the sample sizes are small, e.g. plasma from mouse and rats. Exception is the method from Honda et al. [36] and Xu et al. [38], which both used only 5 µL and picolinyl ester derivatization. Sensitivity in the same range has been achieved with Girard T derivatization (in cell sample) using narrow bore columns [23, 24]. In general, the sensitivity will depend on both the efficiency of the sample preparation and column dimensions. The 2.1 mm ID columns with small particles (e.g. > 2 µm), provide high efficiency separations, but more narrow columns (µm-scale IDs, e.g. nano LC and capillary LC) can be employed when the goal is to enhance sensitivity [23, 24, 49].

4. Unknown Pleasures? Alternative separation approaches for oxysterols

In addition to conventional LC and GC, there are a number of other separation approaches that are less explored regarding oxysterols.

2D GC [50] means to couple two different GC columns in a single system, to enhance chromatographic resolution. The two columns must have different selectivity, and are connected via a modulator. Fractions elute from the first (usually long) column, and are subsequently chromatographed on a second (usually short) column. A large number of chromatograms are generated during an analysis, and dedicated software assembles these into a 2D plot (resemblance of a 2D gel). The combined resolution is in theory the product of the peak capacity of the two columns (in practice, this number is lower). This approach is used in e.g. food and gasoline analysis, but has also been used for mapping sterols [51] (**Figure 5)**. However, the approach is not commonplace, but is commercially available from a number of manufacturers.

2D LC is a similar variant to 2D GC, where two separation columns are coupled, for example hydrophilic interaction chromatography (HILIC) and reverse phase (RP) LC. 2D LC has been used for lipid analysis ([52]), but not for sterols (to the authors´ knowledge). It is worth mentioning here that HILIC highly related to NPLC, has an acceptable stability and is highly MS compatible [53]. Hence, it could be interesting to see if this phase would have promise for oxysterol separations. Although 2D LC can provide very high resolutions, it is arguably less straightforward to operate than 2D GC, as different LC columns are often not compatible with each other's preferred MP solvents. However, it could be interesting so see if 2D LC could fully resolve side chain-hydroxylated oxysterol isomers, by combining methanol:water and acetonitrile:water LC separations in a joint system.

Capillary electrophoresis (CE) and related techniques are characterized by an electric field applied across an open tube/column in which the separation takes place. Compounds are separated by charge and hydrodynamic radius, often with unprecedented resolutions. CE has been used for sterol analysis, using organic solvents (non-aqueous CE = NACE) [54]. Since the approach does not require a solvent pump, it is highly suited for miniaturization/chip separations. However, it remains to be a more technically challenging technique compared to LC and GC.

Open tubular columns (not filled with a particles) are typically used for GC and CE (and related techniques), but are rarely used in LC. However open tubular LC (OTLC) can provide for excellent chromatography and sensitivity. Such columns are typically 10 µm ID, featuring a stationary phase attached to the inner walls, as in GC. OTLC has been demonstrated regarding oxysterols, and Vehus et al. [49] achieved detection limits of 25 attograms (Girard T derivatized 25-hydroxycholesterol). For comparison, previous high sensitivity methods have achieved detection limits in the femtogram range [23, 36]. OTLC is predicted to have a significant role in tomorrow´s liquid separations [55]. However, as with the other techniques presented in this section, it has larger technical challenges, where routine labs cannot be expected to have patience for. This may be resolved when commercial OTLC products/systems become available, although these will perhaps be primarily used for applications with very limited amounts of sample. In addition to the techniques described here, there are other approaches that are rather unexplored regarding oxysterol analysis. For instance, supercritical fluid chromatography (SFC) may be an interesting and useful approach, as SFC is associated with speed and ability to separate isomers. SFC has previously been demonstrated with other sterols and related compounds [56, 57].

5. Conclusions

Oxysterols can be challenging to separate, and some oxysterol pairs such as 24 *R/S* -hydroxycholesterol and 25-hydroxycholesterol, 7α- and 7β-OHC need particularly careful attention to chromatographic separation. Indeed, mass spectrometry can offer an additional level of resolution by differentiating co-eluting compounds by mass and selecting specific ion pairs, e.g. with multiple reaction monitoring methods (MRM). However, quality oxysterol analysis needs quality separations. Regarding oxysterols, LC is becoming increasingly used and developed compared to GC. Newer types of solid support (e.g. core-shell) and stationary phases (e.g. biphenyl) should be further explored for more time efficient separation. Sensitivity is good enough for native oxysterols in serum/plasma if sample sizes are ample (>100 µL), but the inner diameter of the column can be modified to obtain sensitivity gains. Since chromatography is an important aspect in the analysis of oxysterols, we encourage readers to provide details on their chromatographic methods and challenges, to set the stage for faster and more efficient analyses in the future.

6. Acknowledgements

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Table 1 Chromatographic conditions for derivatized oxysterol separation

REF	Derivatization	Column	Dimensions L x ID (mm)	Particle size (µm)	Pore size (Å)	Surface area	Carbon load (%)	Mobile phases and Temperature	Detection limits	Run time (min)	Analytes in retention order
[58]	N-4-(N,N-dimethylamino)phenyl carbamates	Acquity UPLC CSH^{TM} C_{18}	100 x 2.1	1.7	130	185	15	Formic acid (FA) in H_2O/MeOH/ACN, 70 °C	Low nM	13	22R-OHC, 27-OHC, 25-OHC, 24S-OHC, 7β-OHC, 5β,6β-epoxycholestanol, 5α,6α-epoxycholestanol, desmosterol, 7-dehydrocholesterol, lathosterol, cholesterol, cholestenol
[44]	N,N-dimethylglycine	BetaSil C_{18}	100x 2.1	5	100		20	Trichloroacetic acid (TCA)/acetic acid (AA) in H_2O/ACN	2 ng/mL	10	3β,5α,6β triol, 7-keto-OHC
[46]	N,N-dimethylglycine	Betasil C_{18}	100x 2.1	5	100		20	TCA/AA in H_2O/ACN	2 ng/mL		3β,5α,6β triol, 7-keto-OHC
[42]	N,N-dimethylglycine	Kintex^{TM} Biphenyl	50 x 2.1	2.6 Core shell	100	200	11	FA/Ammonium acetate in H_2O/MeOH/ACN, 30 °C	1 ng/mL	8	25-OHC, 24S-OHC, 27-OHC, 4β-OHC, 7α-OHC 7β-OHC, 7-keto-OHC, 3β,5α,6β triol
[59]	N,N-dimethylglycine	Gemini-NX^{TM} C_{18}	100x2	3	110	375	14	Ammonium formate in H_2O/ACN	0.08-0.8 ng/mL	15	3β,5α,6β triol, 7-keto-OHC
[35-37, 60]	Picolinyl ester	Hypersil GOLD C_{18}	150 x 2.1	3	175	220	10	FA in H_2O/MeOH/ACN, 40°C	2-10 fg on column	40	24S-25-epoxy-OHC, 22R-OHC, 24S-OHC, 25-OHC 27-OHC, 7α-OHC, 4β-

											OHC,
[61]	Picolinyl ester	Acquity UPLC BEH C_{18}	100 x 2.1	1.7	130	185	17.7	FA in H_2O/MeOH/ACN, 35 °C	2 ng/mL	11	4α-OHC, 4β-OHC
[38]	Picolinyl ester	Hypersil GOLD C_{18}	50 x 2.1	1.9	175	220	10	FA in H_2O/ACN , 25°C	5 ng/mL	16	24S-OHC/25-OHC*, 27-OHC/7α-OHC/7βOHC*, 4α-OHC, 4β-OHC, cholesterol
[39]	Picolinyl ester	Hypersil GOLD C_{18}	50 x 2.1	1.9	175	220	10	AA in H_2O/ACN, 40 °C	5 ng/mL	15	4α-OHC, 4β-OHC
[48]	Picolinly ester	Kinetex C_{18}	100x 2.1	2.6	100	200	12	FA in H_2O/MeOH, 25 °C	0.5-5 ng/mL	10	24S-OHC, 25-OHC, 27-OHC
[30-34]	Girard P	Hypersil GOLD C_{18}	50 x 2.1	1.9	175	220	10	FA in H_2O/MeOH/ACN		17	24S-OHC, 25-OHC, 27-OHC, 7β-OHC, 7-O-OHC,7α-OHC, 6-OHC
[62]	Girard P	Kinetex C_{18}	50 x 2.1	1.7	100	200	12	FA in H_2O/MeOH/ACN		17	24S-OHC, 25-OHC, 27-OHC, 7β-OHC, 7-O-OHC, 7α-OHC, 6-OHC
[25]	Girard T	ACE C_{18}	150 x 1	3	300	100	9	FA in H_2O/ACN, 40 °C	0.2 nM	20	25-OHC, 24S-OHC, 20α-OHC, 22S-OHC
[23]	Girard T	ACE C_{18}	150 x 0.1	3	300	100	9	FA in H_2O/MeOH	23 pM	40	22R-OHC, 24S-OHC, 25-OHC, 27-OHC, 22S-OHC
[24]	Girard T	ACE C_{18}	150 x 0.3	3	300	100	9	FA in H_2O/MeOH	25 pM	35	22R-OHC, 24S-OHC,25-OHC, 27-OHC, 22S-OHC
[45]	Dimethylaminobutyrate ester	Phenomenex Synergi fusion C_{18}	50 x 2.1	4	100	475	12	FA + ammomium formate in H_2O/ACN	0.5 ng/mL	6	3β,5α,6β triol, 7-keto-OHC

*Coelution

Table 2: Chromatographic conditions for native oxysterol separation

REF	Column	Dimensions L x ID (mm)	Particle size (µm)	Pore size (Å)	Surface area (m2/g)	Carbon load (%)	Mobile phases and Temperature	Detection limits	Run time (min)	Analytes in retention order
[63]	Zorbax Eclipse Plus C18	150 x 2.1	3.5	95	160	9	Ammonium acetate in H_2O/MeOH/ 30°C		30	24-OHC, 27-OHC, desmosterol, cholesterol, lanosterol, cholestanol stigmasterol campesterol, β-sitosterol, sitostanol
[26]	Nucleosil C18 HD	125 x 2	5	120	200	11	Ammonium acetate in MeOH/ACN/ H_2O	25 ng/ml	35	24S-OHC, 27-OHC
[19]	Nucleosil 5-CN	250 x 2	5	100	350	5	Heptane/Propan-2-ol	16 ng	20	Cholesterol, 5,6α-EP, 25-OHC, 7-keto, 7β-OHC and 3β,5α,6β triol
	Ultracarb ODS (20) C18	250 x 2	5	90	370	22	MeOH/ACN	4 ng	20	25-OHC, 3β,5α,6β triol , 7β-OHC, 7-keto, 5,6α-epoxy-OHC, cholesterol
[27]	BetaBasic C18	250 x 2.1	5	150	200	13	Ammonium acetate in MeOH/ACN/H_2O 10°C	30 ng	30	25-OHC, 24-OHC
[64]	Synergi Hydro	250 x 2	4	80	475	19	MeOH/ACN/H_2O 30°C	0.1-0.4 ng/ml	25	3β,5α,6β triol , 7α-OHC, 7β-OHC, 7-keto, β-epoxy-OHC, α-epoxy, 6-keto
[65]	Supleco Ascentis®MS (C8)	100 × 2.1	3	100	450	15	ACN/H_2O/Ammonium acetate	4 ng/ml	7	4β-OHC
[66]	Chromolith SpeedRod RP-18e monolithic	50 × 4.6	2		250	18	MeOH/H_2O	0.1 ng/ml	7	cholestane 3ß,5α,6β-triol, 7-α/β-hydroxycholesterol, 5,6-β-epox-OHC, 5,6-α-epoxy-OHC, 7-ketocholesterol,

										cholesterol.
[67]	Nucleosil HD, C18	250 × 4.6	3	100	350	20	FA in MeOH/ H_2O	10 ng/ml	14	27OHC
[68]	LiChrosorb RP-18	250 ×4	5	100	300	17	MeOH/ACN 30°C	0.2 ng	16	25-OH, 3β,5α,6β triol, 7β-OH, 7-keto,5,6α –epoxy-OHC, cholesterol
[41]	Kinetex C 18	150 ×2.1	2.6	100	200	12	Ammonium acetate in ACN/ /IPA	1 ng/ml	12	60 analytes
[69]	ACQUITY UPLCTMBEH C18	150 × 2.1	1.7	130	185	18	FA in MeOH/H_2O 40°C	54 pg/ml	20	24-OHC, 25-OHC 7-OHC, 4β-OHC and 7-keto cholesterol
[21]	Shimadzu Shim-pack ODS	100 × 3	2.2	8nm	470	20	H_2O/ ACN 50°C		16	24(S)-OHC, 25-OHC, 27-OHC, **7**α, 7β, 4α-,5,6β-epoxy-OHC, 5,6α-epoxy-OHC, 4β-OHC, cholesterol
[70]	ODS AQ C18	150 × 4	5	120	330	14	MeOH/ACN/H2O	100 ng/ml	30	25-OHC, cholestane-3β-5α-6β-triol, 7β-OHC, 7-ketocholesterol, 5,6α-epoxy-OHC, cholesterol.
[71]	Aquasil C18	250 x 4.6	5	100	310	12	ACN/MeOH 25°C	0.5ng	19	7α-, 7β-, 25-OHC, 7-keto, 3β,5α,6β triol, α-epoxy, β-epoxy
[72]	Nova Pack CN HP	300 x 3.9	4	60	120	3	n-Hexane-2-Propanol 32°C	6-70 ng/ml	30	19-OHC, cholesterol, 20 α-OHC, 22(R)-OHC, 24(S) –OHC, 22(S)-OHC, 25-OHC, 5,6 α-epoxy-OHC, 5,6 β-epoxy-OHC, 25(R)-OHC, 7-ketocholesterol, 7β-OHC, 7α-OHC
[73]	NUCLEOSIL® C18	100 x 4	5	100	350	15	FA in MeOH/H_2O/2-propanol	5-135 pg/ml	45	24-OHC, 25-OHC, 27-OHC, 7β-OHC, 7-ketocholesterol
[5]	Supelcosil LC-18-S	250 x 4.6	5	120	170	11	FA in MeOH/H_2O	3.2 ng/ml	45	21 analytes

FIGURE LABELS

Figure 1. GC-MS performance of the method by Diczfalusy and co-workers [13]. Broken lines are unlabeled compounds, and solid lines are deuterated internal standards. Compounds separated (plasma sample) are: I. 7α-hydroxycholesterol, II. 7β-hydroxycholesterol, III. cholesterol-5α,6α-epoxide, IV. cholesterol-5β,6β-epoxide, V. cholestane-3β,5α,6β-triol, VI. 24-hydroxycholesterol, VII. 25-hydroxycholesterol, VIII. 7-oxocholesterol, IX. 27-hydroxycholesterol. All compounds were derivatized with TMS. Reprinted with permission.

Figure 2. GC-MS performance of the method by M T Rodriguez-Estrada and co-workers [15]. The total ion current chromatogram shows a fast GC-MS separation of 1. 7α-hydroxycholesterol, 2. 19-hydroxycholesterol, 3. 7β-hydroxycholesterol, 4. β-epoxycholesterol, 5. α-epoxycholesterol, 6, cholestanetriol, 7. 25-hydroxycholesterol; 8, 7-ketocholesterol. All compounds were derivatized with TMS. Reprinted with permission.

Figure 3. LC-MS performance of the method by S Matysik and co-workers [42]. Selected peaks: **1.** 25-hydroxycholesterol, **2.** 24(S)-hydroxycholesterol, **3.** 27-hydroxycholesterol, **4.** 7β-hydroxycholesterol, **5.** 7α-hydroxycholesterol, **6.** 4β-hydroxycholesterol, **7.** 7-ketocholesterol, **8.** cholestan-3β,5α,6β-triol. All compounds were derivatized with DMG. Reprinted with permission.

Figure 4. The most common derivatizations reaction end products for oxysterol analysis; Girard P, Girard T, Picolinyl ester and N,N-dimethylglycin.

Figure 5. GCxGC-FID performance of the method by Tranchida et al. [51]. The 2D chromatogram is of a commercial sunflower oil («sterol zone»). Compounds are: 1. Cholesterol (methylsterol = DesMe), 2. Brassicasterol (DesMe), 3. Ergosta-5,7,9(11),22-tetraen-3beta-ol (DesMe), 4. Ergosterol (DesMe), 5. 24-methylene-cholesterol (DesMe), 6. Campesterol (DesMe), 7. Campestanol (DesMe), 8. Stigmasterol (DesMe), 9. Ergosta-7-en-3β-ol (DesMe), 10. Clerosterol (DesMe), 11. β-sitosterol (DesMe), 12. Lupeol (dimethylsterol = DiMe), 13. Δ^5-avenasterol (DesMe), 14. Parkeol (DiMe), 15. β-amyrin (DiMe), 16. Δ^7-stigmastenol (DesMe), 17. Δ^7-sitosterol (DesMe), 18. Cycloartenol (DiMe), 19. Δ^7-avenasterol (DesMe), 20. α-amyrin (DiMe), 21. 24-methylene-cycloartanol (DiMe), 22. Citrostadienol (methylsterol = Me), 23. Erythrodiol (Diol). Reprinted with permission.

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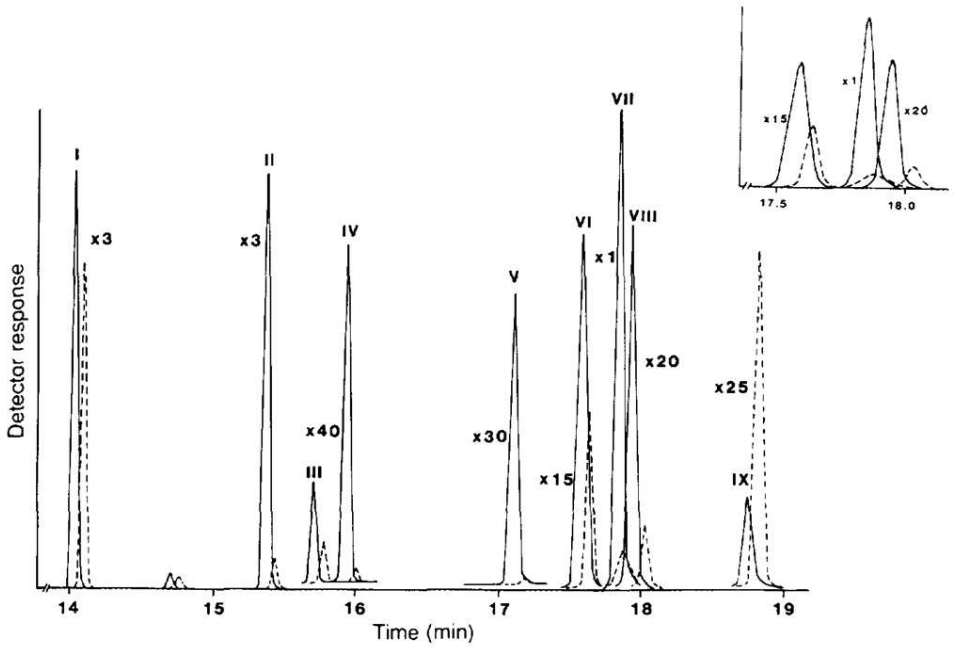

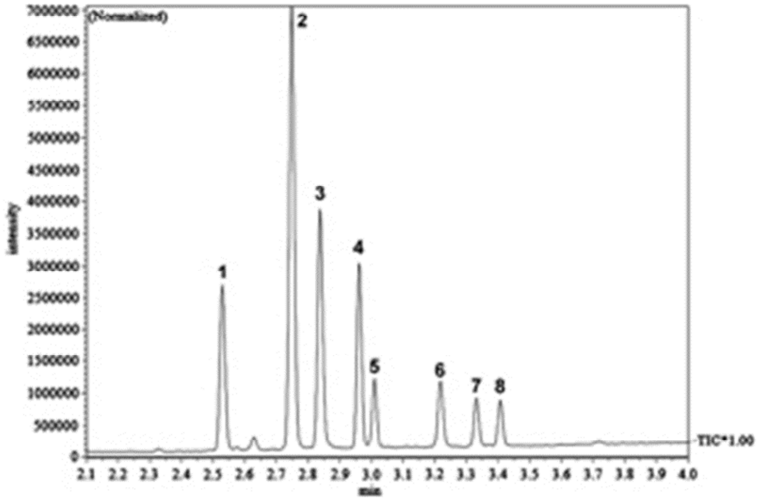

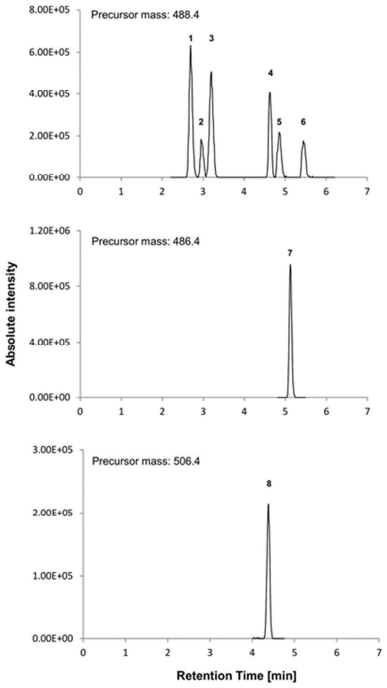

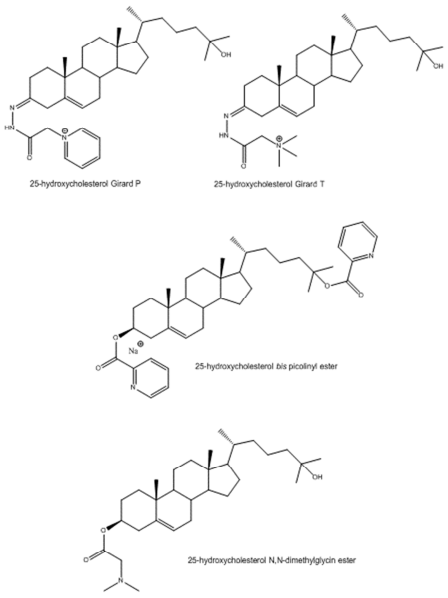

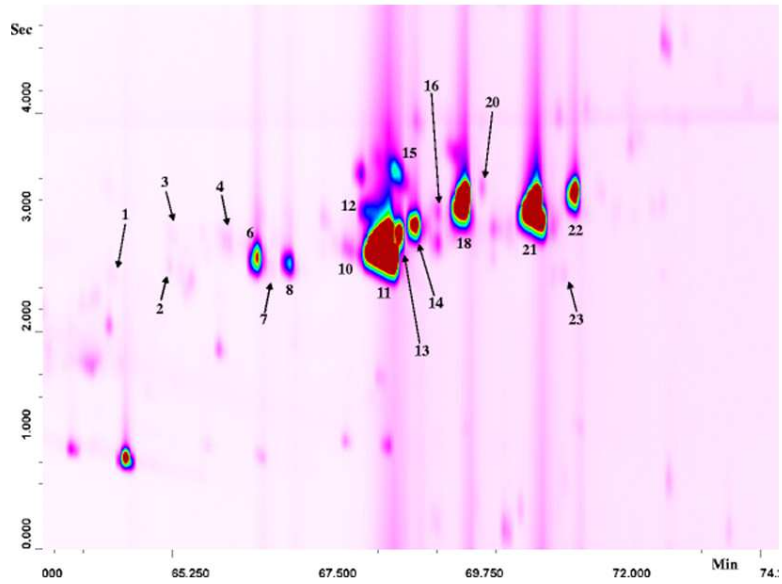

Highlights

- Gas chromatography for oxysterols is well established
- More diversity in liquid chromatography approaches
- Approaches to enhancing speed and selectivity with LC are presented
- Few differences in chromatography between native and derivatized oxysterols
- Alternative approaches to separation of oxysterols exist, but are little explored