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CHROMATOGRAPHY OF OXYSTEROLS

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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.

1 CHROMATOGRAPHY OF OXYSTEROLS

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14 **Highlights**

15 ➢ Gas chromatography for oxysterols is well established

16 ➢ More diversity in liquid chromatography approaches

17 ➢ Approaches to enhancing speed and selectivity with LC are presented

18 ➢ Few differences in chromatography between native and derivatized oxysterols

19 ➢ Alternative approaches to separation of oxysterols exist, but are little explored

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23 **Key words (max 6)**

24   Oxysterols; Chromatography; Separation; Lipids; Sterols
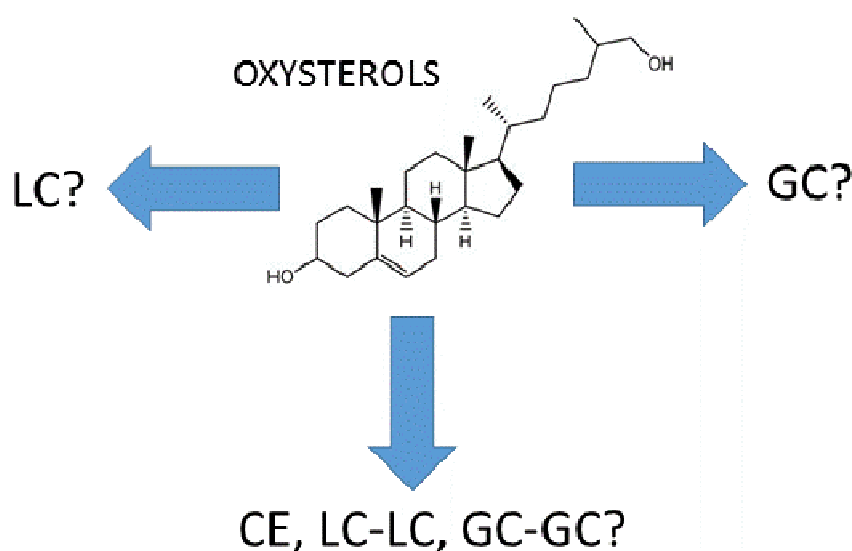
25   **Abstract**

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28   chromatography prior to detection. Here, we will devote attention to the chromatography of
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31   We discuss how these parameters may affect the chromatography, regarding factors such as
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33   oxysterols.

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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

92 interface with LC) [8, 9]. Reduced suppression from other compounds lessens the need for

93 analyte-specific internal standards. GC has been a workhorse for analysis of sterols for well

94 over 50 years [10-12]. For the last couple of decades, a method described by Ulf Diczfalusy

95 and co-workers for oxysterol analysis has been highly influential [13]. For measurements of

96 the analytes in human plasma, the authors separated 7α- and 7β-hydroxycholesterol, 7-

97 oxocholesterol, cholesterol-5α,6α-epoxide, cholesterol-5β,6β-epoxide, cholestane-3β,5α,6β-

98 triol, 24-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol and used MS

99 for detection (**Figure 1**). The analytes were derivatized with trimethylsilyl (TMS) to enhance

100 volatility as required by GC. The authors employed a 30 meter long column, featuring a HP-

101 5MSM ((5%-phenyl)-methylpolysiloxane) film, which is described as a non polar "general

102 purpose" stationary phase, where separations are mainly based on differences in boiling

103 points. A temperature gradient was employed and the MP (helium) was held at 0.8 mL/min

104 (35 cm/second), which is close to the optimal column efficiency for the MP and column inner

105 diameter (ID) (0.25 mm). Using these conditions, the authors obtained peaks with widths of

106 about 15 seconds, with all the analytes being detected within 19 minutes.

107 This method has been cited several hundred times, and more importantly, has been

108 reproduced/re-used in a substantial number of studies. Quite remarkably, few major

109 significant modifications to this GC method have been reported. Some researchers have

110 reduced the analysis time; for determining serum cholestane-3β,5α,6β-triol as a biomarker for

111 Niemann-Pick type C disease (NPC) diagnosis, Kannenberg et al. performed analyses below

112 10 minutes per sample. The authors employed a trifluoropropylmethyl polysiloxane phase, a

113 mid-polar stationary phase, which is promoted as being ideal for separating positional isomers

114 [14]. A most notable reduction of analysis time was shown by Maria T. Rodriguez-Estrada

115 and co-workers, who performed analysis of cholesterol oxidation products using "fast gas

116 chromatography" [15]; a shorter (10 m) column with a narrower ID (0.1 mm) and thinner

117 stationary phase film (0.1 µm thickness) allowed for separation (resolution (Rs)>1.2) of 7α-

118 hydroxycholesterol, 19-hydroxycholesterol, 7β-hydroxycholesterol, β-epoxycholesterol, α-

119 epoxycholesterol, cholestanetriol, 25-hydroxycholesterol, 7-KC in 3.5 minutes (**Figure 2**). It

120 should be noted that a narrow ID and thin film allow for improved efficiency, which can be

121 essential for obtaining resolution when the column length is decreased (reduces analysis time,

122 but affects the separation). The fast analysis time of this method is roughly 5-10 times faster

123 than that described in most related papers. The flow rate was 0.41 mL/minute, which

124 corresponds to a linear velocity of 43 cm/second (narrow columns can be operated at higher

125 linear velocities without dramatic decreases in efficiency). However, substantial efforts

4

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

5

160 *3.2 RPLC: a workhorse with possibilities for different selectivity*

161 RP stationary phases are hydrophobic and are typically alkyl chains. The oxysterols, both

162 native and derivatized are most commonly separated using octadecyl alkyl chain (C18)-

163 bonded silica stationary phases, followed by C8-bonded silica columns (**Tables 1 and 2**). The

164 analytes are expected to elute according to hydrophobicity (although RPLC is far more

165 complex than commonly perceived [20]). With RPLC, the more polar side chain oxygenated

166 oxysterols elute before ring-oxidized sterols followed by more non-polar sterols [19, 21]).

167 C18 columns generally provide the same retention order for oxysterols (when using similar

168 MPs) regardless of the derivatization reagent (derivatization in LC is discussed in some more

169 detail below). This implies that selectivity differences are typically not attributed to the

170 derivatization reagent. However, RPLC can have somewhat surprisingly degrees of selectivity

171 options. Shan et al. compared oxysterol separations regarding two solvent systems,

172 acetonitrile:water and methanol:water, on C8 and C18 columns [22]. They showed that even

173 with same MP the chromatographic mobility and selectivity between C8 and C18 columns are

174 considerably different. The C8 column was able to resolve several oxysterol pairs, including

175 7α- and 7β-hydroxycholesterol, which were inseparable on the C18 column using a similar

176 MP. Compared to using methanol:water, using acetonitrile:water with a C18 column provided

177 improved resolution of oxysterol pairs 27-hydroxycholesterol/3β,5α,6β-triol, 24R-

178 hydroxycholesterol/20α-hydroxycholesterol, 7α-hydroxycholesterol/7β-hydroxycholesterol,

179 and 7-ketocholesterol/3β-OH-6-one. Roberg-Larsen et al. observed that employing an

180 acetonitrile-based MP and C18 allowed separation of 20-hydroxycholesterol and 27-

181 hydroxycholesterol, while separation of 27-hydroxycholesterol and 24S-hydroxycholesterol

182 was not possible with the same conditions [23]. The opposite was observed when using

183 methanol-based MP (in the final section we will discuss an approach that may allow these two

184 systems to be combined). Roberg-Larsen et al. has observed highly similar RPLC oxysterol

185 separations in microbore LC, capillary LC and nano LC [23-25] (selectivity is rarely affected

186 by column diameter).

187

188 24S-hydroxycholesterol and 25-hydroxycholesterol can also be challenging to separate.

189 Debarber et al. separated 24R- and 24S-hydroxycholesterol by modifying a method by

190 Burkard and coworkers [26] using a methanol:acetonitrile:water MP (45:40:35) and a column

191 temperature of 55 °C [27]. Changing to methanol/acetonitrile/water (14:0.6:1) and a column

192 temperature of 10 °C same authors demonstrated separation of 24-hydroxycholesterol from

193 25-hydroxycholesterol within short (6.5 min) time. The later method reversed the retention

194   order of two oxysterols to 24-hydroxycholesterol followed by 25-hydroxycholesterol.

195   However, the later method did not baseline separate the two oxysterols.

196

197   Great efforts have been made to ensure that the analytes do not interact with the particles in

198   which the stationary phase is attached, as e.g. silica particles cause secondary interactions and

199   may perturb the separation (e.g. cause band broadening and tailing). Avoiding interactions

200   with the particles can be done by efficient rest-silanization and adding functional groups at the

201   trunk of the main stationary phase for steric hindrance. In addition, the carbon loading of the

202   particles is crucial for oxysterol separations. For instance, otherwise high quality columns

203   which featured lower carbon loads were unable to provide selective separation of side-chained

204   oxysterols [25].

205

206   To improve chromatographic efficiency further, the size of the particles may be reduced;

207   today sub-µm particles are common, while 3-5 µm particles were standard about a decade

208   ago. The Hypersil GOLD[TM] column is a familiar column in oxysterol RPLC, both in UHPLC

209   (sub 2 µm particles) and regular HPLC (3 µm particles), typically with a 2.1 mm ID. The

210   Hypersil GOLD[TM] columns are endcapped silica-based columns, with a high hydrophobicity

211   and medium shape selectivity and polar surface activity. (For column classification see [28]

212   and [29]) This column material has been used for chromatographing both Girard P- and

213   Picolinyl ester derivatized (PED) oxysterols [30-39]. This column seems to not be compatible

214   with Girard T derivates (Rs >1.1, data not published). However, sub-µm particles cause

215   higher back-pressures. Therefore, a highly attractive alternative has been the use of core shell

216   particles. Core shell particles have a solid core and porous shell, that gives high efficiency and

217   fast separations with low back pressure compared to traditionally porous particles [40]. Core

218   shell particles provide similar efficiencies to sub-µm particles. A well-known example of core

219   shell particles for oxysterol analysis is that by McDonald et al. who chromatographed 62

220   different sterols, oxysterols and secosteroids from human plasma using two different LC and

221   one GC method. Both the LC methods used core shell particles, with the side-chain

222   oxysterols eluting in 7.5min (total run time 12min) [41].

223   A notable exception from using octyl chain stationary phases is by Silke Matysik and co-

224   workers who employed a biphenyl phase [42]. Biphenyl stationary phases typically provide

225   increased retention and can have a different selectivity compared to traditional C18/RP

226   phases, as it can provide both $\pi-\pi$ interactions and higher hydrogen bonding capacities [43].

227   In addition to featuring a different phase, the column employed was packed with core shell

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

230

231 *3.3 Effect of derivatization on oxysterol chromatography*
232 Derivatization of oxysterols is used to enhance sensitivity in MS detection by incorporation of
233 a charge group into the oxysterol. Derivatization of oxysterols can be used with both ESI-MS
234 and atmospheric pressure chemical ionization (APCI)-MS. In addition to enabling enhanced
235 sensitivity, derivatization can also make the sterols more soluble in MPs commonly used in
236 RPLC. Derivatization can also prevent adsorption of the hydrophobic sterols on narrow ID
237 fused silica tubing in use in sensitive nano LC-based systems [7].   Other benefits of
238 derivatization are more easily interpretable MS2 spectra, as fragmentation of the derivatized
239 group usually gives more specific fragmentation [7].

240

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

251

252 Regarding chromatographic performance, there are small differences in the behavior of the
253 derivatized or native oxysterols. Cha et al. [48] has analyzed both native oxysterols from
254 serum samples as silver adducts and picolinyl ester derivatized (PED) oxysterols from CFS.
255 Although the chromatograms look very different regarding analysis time, the analysis is
256 performed on two different reversed phase columns; An ACE C18 (3 µm, 150 mm x 2.1 mm
257 ID) and a Kinetex C18 (2.6 µm, 100 mm x 2.1 mm ID, core shell). Although these columns
258 has approximately the same hydrophobicity, they have different shape selectivities and polar
259 surface activities [28] and most importantly, different solid supports (fully porous vs. core-
260 shell). It would be interesting to compare the separation of the PED with McDonald et al.
261 [41], and the native oxysterol separation with Roberg-Larsen et al [24], which both uses the

8

262 same columns on native and Girard T derivatized oxysterol, respectively. McDonald's

263 oxysterol-ammonium adducts shows similar chromatography and elutes in the same retention

264 window as Cha's PED, while Roberg-Larsen's Girard T derivates elutes in the same retention

265 window as Cha's native sterol.

266

267 *3.4 Dimensions and sensitivity*

268 The most popular column dimension used in oxysterol analysis is the 2.1 mm ID format.

269 Detection limits for native oxysterols and all the types of derivates are in low ng/mL, suitable

270 for analyzing oxysterols in plasma. However, most of the applications use more than 50 µL

271 plasma or serum in their sample preparation. The relatively high sample volume for the other

272 methods can be challenging if the sample sizes are small, e.g. plasma from mouse and rats.

273 Exception is the method from Honda et al. [36] and Xu et al. [38], which both used only 5 µL

274 and picolinyl ester derivatization. Sensitivity in the same range has been achieved with

275 Girard T derivatization (in cell sample) using narrow bore columns [23, 24]. In general, the

276 sensitivity will depend on both the efficiency of the sample preparation and column

277 dimensions. The 2.1 mm ID columns with small particles (e.g. > 2 µm), provide high

278 efficiency separations, but more narrow columns (µm-scale IDs, e.g. nano LC and capillary

279 LC) can be employed when the goal is to enhance sensitivity [23, 24, 49].

280

281

282 **4. Unknown Pleasures? Alternative separation approaches for oxysterols**

283 In addition to conventional LC and GC, there are a number of other separation approaches

284 that are less explored regarding oxysterols.

285 2D GC [50] means to couple two different GC columns in a single system, to enhance

286 chromatographic resolution. The two columns must have different selectivity, and are

287 connected via a modulator. Fractions elute from the first (usually long) column, and are

288 subsequently chromatographed on a second (usually short) column. A large number of

289 chromatograms are generated during an analysis, and dedicated software assembles these into

290 a 2D plot (resemblance of a 2D gel). The combined resolution is in theory the product of the

291 peak capacity of the two columns (in practice, this number is lower). This approach is used in

292 e.g. food and gasoline analysis, but has also been used for mapping sterols [51] (**Figure 5**).

293 However, the approach is not commonplace, but is commercially available from a number of

294 manufacturers.

295

296 2D LC is a similar variant to 2D GC, where two separation columns are coupled, for example
297 hydrophilic interaction chromatography (HILIC) and reverse phase (RP) LC. 2D LC has been
298 used for lipid analysis ([52]), but not for sterols (to the authors´ knowledge). It is worth
299 mentioning here that HILIC highly related to NPLC, has an acceptable stability and is highly
300 MS compatible [53]. Hence, it could be interesting to see if this phase would have promise for
301 oxysterol separations. Although 2D LC can provide very high resolutions, it is arguably less
302 straightforward to operate than 2D GC, as different LC columns are often not compatible with
303 each other's preferred MP solvents. However, it could be interesting so see if 2D LC could
304 fully resolve side chain-hydroxylated oxysterol isomers, by combining methanol:water and
305 acetonitrile:water LC separations in a joint system.

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

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

329

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

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$_2$O/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$_2$O/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$_2$O/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$_2$O/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$_2$O/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$_2$O/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$_2$O/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$_2$O/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$_2$O/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$_2$O/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$_2$O/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$_2$O/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$_2$O/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$_2$O/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$_2$O/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$_2$O/ACN	0.5 ng/mL	6	3β,5α,6β triol, 7-keto-OHC

*Coelution

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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,

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										cholesterol.
[67]	Nucleosil HD, C18	250 × 4.6	3	100	350	20	FA in MeOH/ H$_2$O	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$_2$O 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$_2$O/ 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$_2$O/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$_2$O	3.2 ng/ml	45	21 analytes

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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.
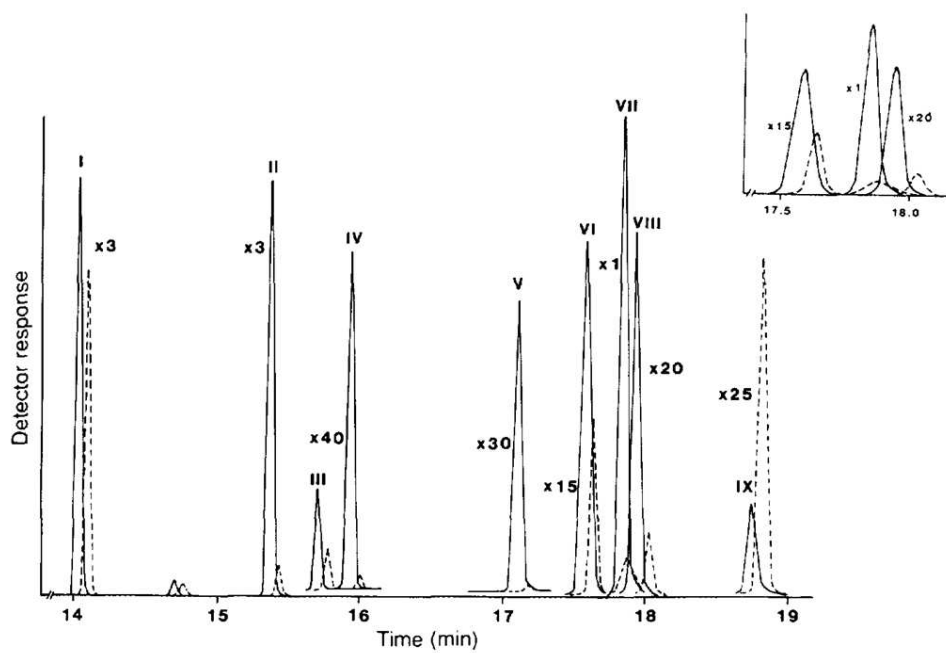
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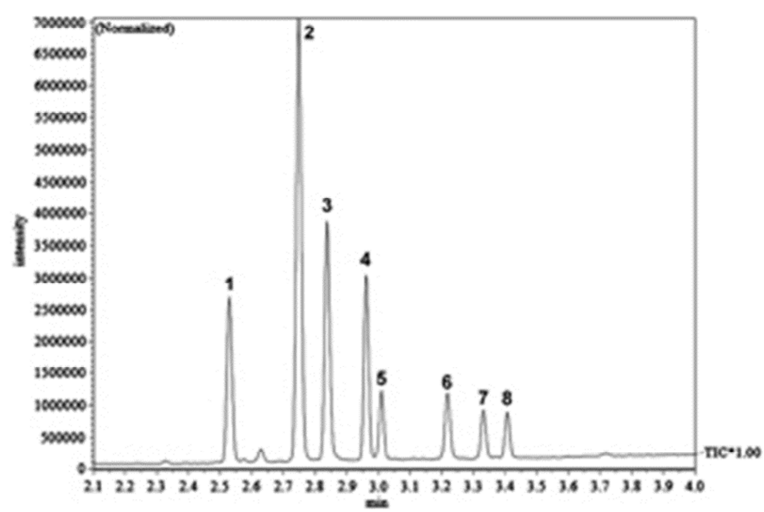
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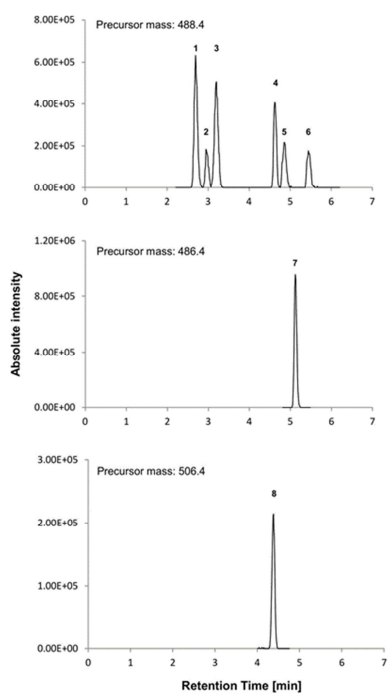
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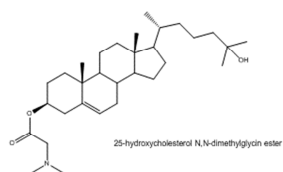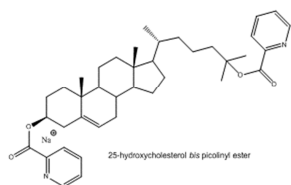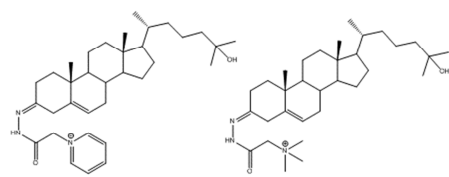
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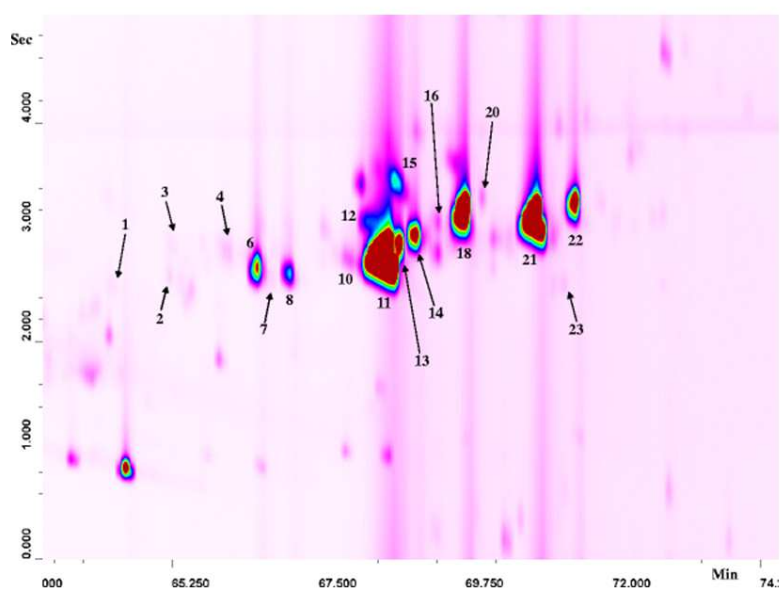25-hydroxycholesterol Girard P

25-hydroxycholesterol Girard T

25-hydroxycholesterol *bis* picolinyl ester

25-hydroxycholesterol N,N-dimethylglycin ester

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