Exploring selectivity and sensitivity for oxysterol measurements using liquid chromatography-mass spectrometry (LC-MS)

Stian Kjønnås Solheim

Thesis for the Master's
Degree in chemistry
60 study points

Department of Chemistry
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Abstract

Side-chained oxysterols are associated with a plethora of biological functions (*e.g.* cell signaling and development) but are also biomarkers for several diseases. Oxysterols in biological samples may be present in low concentrations (ng/mL) and are neutral isomers that need to be chromatographed before detection by mass spectrometry (MS). This is a difficult task, resulting in run times lasting 15-45 minutes and is therefore rather impractical for diagnostic screening. Different stationary phases (SP), organic solvents and adjustable conditions (*e.g.* flow rate, temperature and mobile phase compositions) were explored to improve the analysis time and resolution (R_S) for separation of side-chained oxysterols.

A new method was developed for separation of the side-chained oxysterols 22R-, 24S-, 25-, 27- and 22S-hydroxycholesterol (OHC) within a 5 minutes analysis time and $R_S \approx 1.5$. This was achieved using an ACE SuperPhenylHexyl (SPH) 2.1 mm inner diameter (ID) column with 2.5 µm core-shell particles. The flow rate was 900 µL/min with a column temperature of 45 °C and a mobile phase (MP) composition consisting of H_2O/methanol/acetonitrile 60/8.5/31.5 (*v/v/v*) with 0.1% formic acid.

The ACE SPH particles was repacked into columns with more narrow ID (0.5, 0.3 and 0.1 mm ID) for increased sensitivity. The column that separated the oxysterols with highest resolution ($R_S \geq 1$) was the 0.1 mm ID column (with ACE SPH particles) where column efficiency was almost the same as the commercial 2.1 mm ID ACE SPH column. By reducing the column ID from 2.1 mm to 0.1 mm a 440 times increase in sensitivity could theoretically be obtained. However, the resolution and analysis time were not acceptable with the 0.1 mm ID column and which is why this column dimension was not investigated further.

Implementation of the on-line automatic filtration and filter back-flush solid phase extraction system (AFFL-SPE) allowed injection of large volumes (*e.g.* 100 µL instead of 1 µL) and on-line sample clean up. This made it possible to use the 2.1 mm ID ACE SPH column and detect the oxysterols from 5 µL plasma with an analysis time of 5 minutes and $R_S = 1.2$. Hence, the method developed in this study have the potential to be applied in a diagnostic screening setting of oxysterols using only a single drop of blood.

Preface

The work presented in this thesis was performed at the Department of Chemistry at the University of Oslo from January 2016 to March 2018. My supervisors have been Professor Elsa Lundanes, Associate Professor Steven Ray Haakon Wilson and Postdoc Hanne Røberg-Larsen. I would like to thank them all, but especially Postdoc Hanne Røberg-Larsen for all her help and guidance, both for my bachelor project and my master thesis. She has been a motivator and a good help in times with adversity.

I would like to thank all fellow students at the research group of Bioanalytical chemistry for all the fun, motivation and cooperation. Everybody have contributed to a good and inclusive working environment that has been essential during my time here. A special thanks to by office buddies Sunniva Furre Amundsen and Ramneet Kular that have become good friends over the past two years. They have supported me in times when motivation and inspiration has been absent and I am very grateful for getting to know both of you.

I would also like to thank my family, especially my mom and big brother for the motivation and love even though they have no idea of what this thesis is about. Still, I do not think I would have manage to go through it all this without them. Also, a huge thanks to all my friends for all the support throughout my degree and for making sure I have a social life outside the University. A very special thanks to my partner Kenneth André Eilertsen for being in my life, for all the motivation, love, support and care through my education and in our daily life.

Oslo, Norway, March 2018

Stian Kjønnås Solheim

Abbreviations

Abbreviation	Definition
22R-OHC	22(R)-Hydroxycholesterol
24S-OHC	24(S)-Hydroxycholesterol
25-OHC	25-Hydroxycholesterol
27-OHC	27-Hydroxycholesterol
22S-OHC	22(S)-Hydroxycholesterol
ACN	Acetonitrile
AFFL-SPE	Automatic filtration and filter back-flush with solid phase extraction
A_S	Asymmetry factor
CSF	Cerebrospinal fluid
D	Diffusion coefficient
Da	Dalton (1/12 of the mass of a ^{12}C atom)
DC	Direct current
DF	Downscaling factor
DMG	N,N-Dimethylglycine
d_p	Particle diameter
EI	Electron ionization
EIC	Extracted ion chromatography
ERα	Estrogen receptor positive
ERβ	Estrogen receptor negative
ESI	Electrospray ionization
EtOH	Ethanol
FA	Formic acid
FSC	Fused silica capillary
GC	Gas chromatography
H	Plate height
HPLC	High performance liquid chromatography
ID	Inner diameter
k	Retention factor

kw	Retention factor when water is used as MP
L	Length of a column
LC	Liquid chromatography
LC-MS	Liquid chromatography coupled to mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
LXR	Liver X receptor
m/z	Mass to charge ratio
MeOH	Methanol
MP	Mobile phase
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
N	Plate number
N'	Avogadro number
NA	Not available
NP-LC	Normal phase liquid chromatography
NPC1	Niemann-Pick C1
OHC	Oxysterol *and* hydroxycholesterol
P	Pressure
PEEK	Polyether ether ketone
r	Radius of a molecule
R	The gas constant
RF	Radio frequency
RP	Reversed phase chromatography
RPLC-MS	Reversed phase liquid chromatography mass spectrometry
Rs	Resolution
RSD	Relative standard deviation
S/N	Signal to noise ratio
SP	Stationary phase
SPE	Solid phase extraction

SPH	SuperPhenylHexyl
STD	Standard deviation
T	Temperature
TIC	Total ion current
t_R	Retention time
TripleQ-MS	Triple quadrupole mass spectrometer
u	Linear velocity (flow rate)
v/v* or *v/v/v	The volume to volume ratio
$w_{av,\ 0.5}$	The average peak width measured at half height of both of the peaks
α	Separation factor
σ	Standard deviation
η	Viscosity
κ	Constant
Φ	Volume fraction of the organic solvent in the MP

Definitions

Term	Definition
Base peak	The most intense peak in a chromatogram.
***Cis* (or Z)**	Describes the position of substituents on either side of a double bond. *Latin*; on this side.
Co-elution	When two or more analytes elute too close to each other to be identified.
Core-shell particles	Particles with a solid core which is coated with a thin totally porous layer.
Fronting	The front half of the peak is wider than the back part.
Gradient elution	An elution where the mobile phase composition is changed during the analysis.
Grounding union	Grounding stainless steel 1/16" union.
H_2O	In this thesis, always type 1 water.
In vitro	Latin for "in glass" (not found endogenously).
In vivo	Latin for "within the living" (found endogenously).
Isocratic elution	An elution with a constant mobile phase composition during the analysis.
Method end-time	A pre-set time for the method (analysis) to end (both the LC and MS ends at this time).
nanoViper	nanoViper™ Fingertight Fittings.
Relative abundance	The intensity relative to the base peak.
Smoothing	A software editing feature to decide how many data points a chromatographic peak should be displayed with.
Tailing	The back half of the peak is wider than the front part.

Totally porous particles	Spherical particles with porous pores all the way through.
Total ion current (TIC)	The summed intensity across the entire range of masses being detected at every point in the analysis.
***Trans* (or E)**	Describes the position of substituents on either side of a double bond. *Latin*; on the other side.
t_M	The time it takes (from injection to detection) for a compound with no interactions towards the SP.
Viper SSF	Viper™ Stainless Steel Fingertight Fittings.

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1 Introduction

1.1 Oxysterols

Oxysterols, also called hydroxycholesterols (OHC), are products of the cholesterol biosynthesis process or oxidized 27-carbon derivatives of cholesterol. Oxysterols derive from enzymatic or non-enzymatic oxidation (autoxidation) of cholesterol [1-3]. The oxysterols with hydroxyl group on the sterol ring are usually formed through autoxidation, while the side-chained oxysterols (hydroxyl-group on the side chain) are usually formed by enzymatic oxidation [4]. The oxysterols with the hydroxyl group on the ring structure (*e.g.* 4β-hydroxycholesterol) behave very similar to cholesterol and are therefore not assessed in this thesis [5, 6]. Side-chained oxysterols (illustrated with 25-OHC in ***Figure 1)*** on the other hand are associated with several biological functions (*e.g.* the effect on critical genes in the cholesterol metabolism [7]), their ligand binding properties as well as their behavior within the cell membrane [8, 9], and which are some of the reason that side-chained oxysterols are the main focus in this thesis. Other biological roles associated with the side-chained oxysterols will be discussed later in ***Section 1.1.1***.

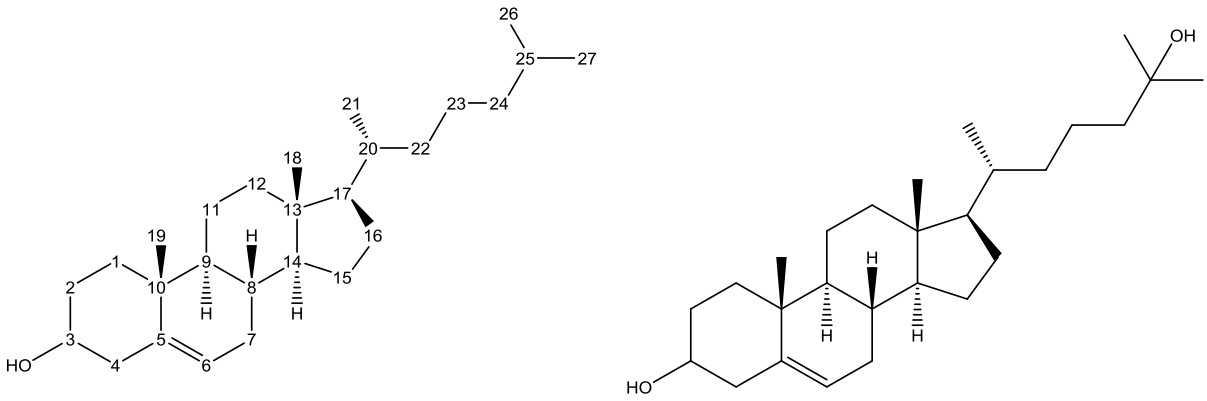


Figure 1: *Cholesterol (left) with IUPAC numbering of carbons. 25-OHC (right) with a hydroxyl group on position 25 on the side-chain.*

An overview of the trivial and systematic names for the oxysterols investigated is given in ***Table 1*** together with the enzymes that converts cholesterol to the different oxysterols**.** Their trivial names (*e.g.* 24S-OHC) will be used. 27-OHC is the most commonly used trivial name for cholest-5-ene-3β,26-diol in the context of medicine and cancer [10], although the correct

name would be 25R,26-hydroxycholesterol [11]. The norm of applying 27-OHC in medicine and cancer is the reason it will be applied in this thesis as well. The side-chained oxysterols studied are illustrated in ***Figure 2***.

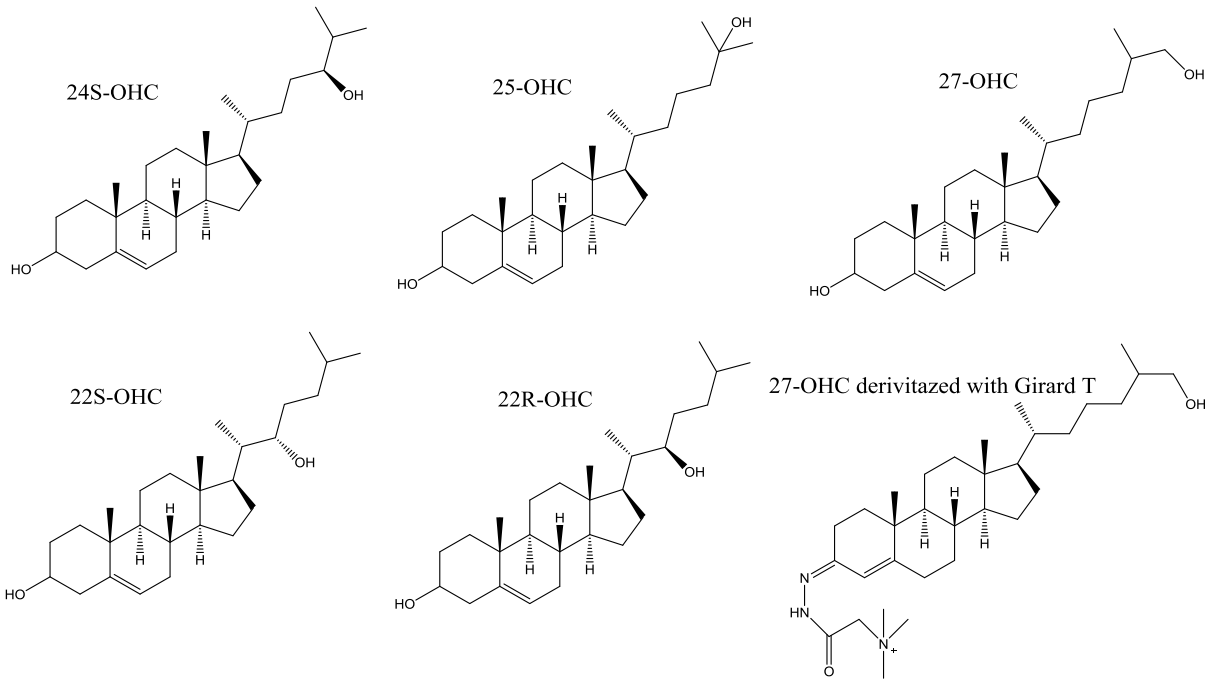


Figure 2: *The side-chained oxysterols investigated; 24S-, 25-, 27-, 22S- and 22R-OHC with the structure of 27-OHC after derivatization with Girard T reagent.*

The oxysterols present at the highest levels in human serum are 27-, 24S-, 7α- and 4β-hydroxycholesterol [2]. Two of these oxysterols are side-chained, 27-OHC and 24S-OHC. These are studied here along with three other side-chained oxysterols; 22R-, 22S - and 25-OHC.

Table 1: *The trivial and systematic name of oxysterols.*

Trivial name	Systematic name	Reaction enzyme
24S-hydroxycholesterol	Cholest-5-ene-3β,24S-diol	CYP46A1
25-hydroxycholesterol	Cholest-5-ene-3β,25-diol	CH25H
22S-hydroxycholesterol	Cholest-5-ene-3β,22S-diol	Not known
22R-hydroxycholesterol	Cholest-5-ene-3β,22R-diol	CYP11A1
27-hydroxycholesterol	Cholest-5-ene-3β,26-diol	CYP27A1

1.1.1 Biological roles related to oxysterols

By introducing a hydroxyl group in the cholesterol molecule, the biological half-life of the molecule will, with a few exceptions, be reduced. This leads to secretion of the oxysterols from the cells to further oxidation to water-soluble bile acids in the liver [12-14]. The rapid degeneration and excretion of oxysterols are made easier by their physical properties, enabling them to pass lipophilic membranes (due to their high polarity) and spread inside the cell at a faster rate than cholesterol itself [5, 6, 12]. This makes them important as a way to transport cholesterol molecules in the metabolism [2, 13].

Oxysterols are present at relatively small concentrations. However, oxysterols are considered to have major effects on biological functions. The ***Figure 3*** shows an overview of the major biological functions of the side-chained oxysterols, and includes *e.g.* metabolic intermediates [15], modulators of cell permeability [16, 17], atherogenic agents (substance or process that cause atherosclerosis) [18, 19], regulation of gene expression [20], regulation of cholesterol homeostasis [4, 21] and their role in cellular signaling [22, 23]. Oxysterols have been suggested to mediate the effects of the membrane protein Patched and hence the membrane protein Smoothened in the Sonic Hedgehog signaling pathway [24, 25]. This signaling pathway is one of the key regulators in the human embryologic development and is central in the development of many types of cancer [26-28].

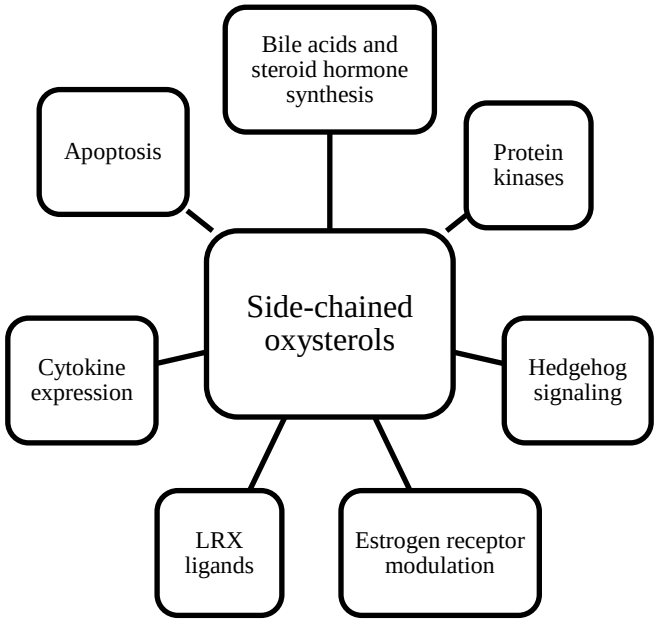


Figure 3: *Schematic overview of the major functions of side-chained oxysterols.*

Yao *et al.* reported that 22R-OHC protect the neurons in the brain against β-amyloid-induced cell death and is found in lower concentration in the brain of patients with Alzheimer's disease [29]. Moreover, 22R-OHC together with 24S- and 27-OHC are potent activators of the liver X receptors (LXR), which act as cholesterol sensors [9, 30]. LXR reacts to elevated sterol concentration and transcribe genes that controls transport, catabolism and elimination of cholesterol [31]. 22R-OHC can also influence the development of metabolic disorders (*e.g.* hyperlipidemia and atherosclerosis).

27-OHC and 24S-OHC are not only potent activators of the LXR, but have also several other biological functions. 27-OHC is a selective estrogen receptor α modulator [32, 33], an intermediate in bile acid production [14, 33] and has also been closely related with estrogen receptor positive (ER+) breast cancer [34, 35]. 24S-OHC has been proven to be present in lower concentration in plasma for Niemann-Pick C1 (NPC1) disease (a rare progressive neurodegenerative disorder disease) [36], and which is similar to those reported for subjects with Alzheimer's disease or neuroinflammation [37].

Cytokines are proteins that are important in cell signaling and they act through receptors. They are especially important in the immune system, and recent studies have shown that macrophages (a type of white blood cells) can secrete high levels of 25-OHC when stimulated

by bacteria or viruses and which indicates that 25-OHC plays an important role in the immune regulation [38, 39]. 25-OHC is a potent activator of the Hedgehog signaling pathway [40].

It has been reported that 22S-OHC act as an antagonist for the LXR by blocking the access to the LXR receptor, thereby the possibility to activate it [41]. 22S-OHC is not naturally formed *in vivo*, as no known enzyme to generate it exists [41].

There are several other oxysterols in the human body where the hydroxyl group is attached to the side-chain as well as on the ring structure (*e.g.* 7α- and 4β-OHC), but the focus is on the five side-chained oxysterols 22R-, 22S-, 25- 27-, and 24S-OHC. Since oxysterols have different biological roles in the human body ***(Table 2)*** it is essential to be able to measure the levels of each individual oxysterol to understand how they can affect the physiological functions in humans.

Table 2: *A summary of some of the biological functions of hydroxycholesterols.*

Trivial name	Biological function (some examples)
24S-hydroxycholesterol	Activator of the LXR, cholesterol homeostasis in the brain, a biomarker for NPC1, Alzheimer's and neuroinflammation.
25-hydroxycholesterol	An immune regulator and a potent activator of the Hedgehog signaling pathway.
22S-hydroxycholesterol	Antagonist for the LXR.
22R-hydroxycholesterol	An activator of the LXR, can protect neurons against β-amyloid-induced cell death, are a potential biomarker for Alzheimer's.
27-hydroxycholesterol	An activator of the LXR, a biomarker for ER+ breast cancer, a selective estrogen receptor α modulator.

1.2 Determination of oxysterols

When monitoring the physiological status of a human (*i.e.* someone's health status), the standard body fluids used are urine, blood (serum or plasma) and cerebrospinal fluid (CSF). The ease of collection and representation of catabolomic/metabolomics favors urine as fluid. The CSF reflects the brain metabolism and blood can reveal the anabolic, metabolic and catabolic progresses of an organism [42]. In *e.g.* inborn errors screening [43], only a drop of blood is used to monitor several diseases. In the review presented by Griffiths *et al.* [44], the determination of oxysterols in plasma was done by using 50 - 200 µL plasma during the sample preparation. In this thesis, with the inborn error screening program in mind, only 5 µL of plasma will be used, and to achieve this, several challenges need to be addressed.

Concentrations of oxysterols in plasma are in the low ng/mL range [3] and they are neutral compounds. Cholesterol is the most abundant sterol in plasma, hence analyzing cholesterol metabolites (oxysterols) present at levels 3 orders of magnitude below that of cholesterol can be challenging as cholesterol and oxysterols are competing to be identified and distinguished in the same detector. Furthermore, the oxysterols in this study are isomers (*i.e.* same mass, different structure), with a molar mass of 402.65 g/mol (exact mass 402.35 g/mol). The difference between the oxysterols studied in this thesis is the location of the hydroxyl group on the side-chain; hence it is important that they are separated in order to be detected at different times. The emphasis in this thesis will therefore be on the separation of oxysterols before detection. The separation technique is explained in further detail in ***Section 1.3.***

Since the oxysterols are neutral compounds, they are less compatible with *e.g.* electrospray ionization (ESI). The ESI and MS are explained in further details in ***Section 1.6***. By derivatizing the oxysterols (*e.g.* using Girard T reagent to get a charge on the analyte, as illustrated in ***Figure 2***), the efficiency of the ionization is improved, yielding a higher intensity in MS detection (improved sensitivity). In this thesis, derivatization was carried out with the Girard T reagent. Another derivatization reagent that has frequently been used for these analytes is the Girard P reagent, but in our hands, Girard T has provided a better performance with regards to separation and detection. The derivatization reaction is divided into two steps, where the first is the oxidation of the hydroxyl-group on position three of the ring structure as illustrated in ***Figure 4***.

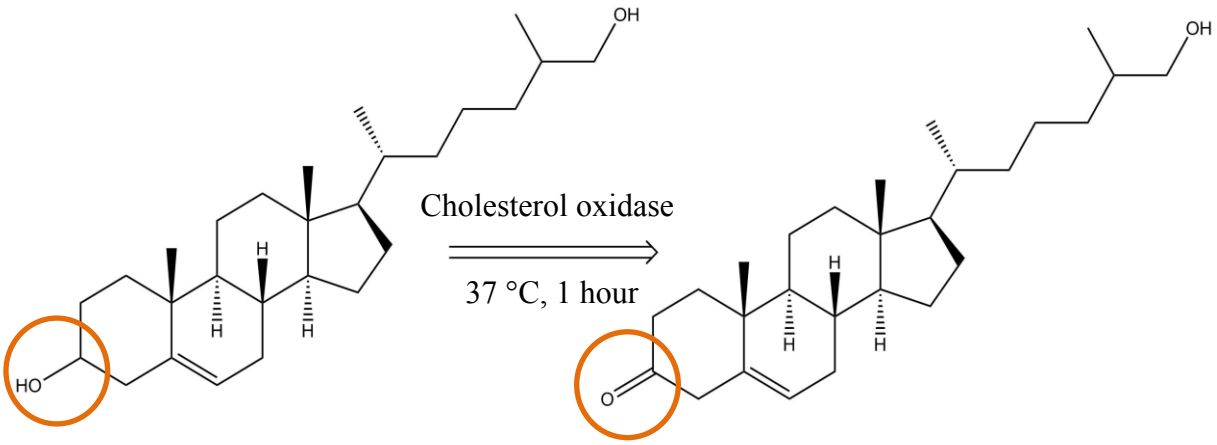


Figure 4: *27-OHC enzymatically oxidized with cholesterol oxidase, illustrated with circle.*

The next step is to derivatize the hydroxycholesterol and this is done by adding the Girard T reagent to the 3-oxo-4-ene group created after oxidation. The final product with *m/z* 514.4 for detection is shown in ***Figure 5***.

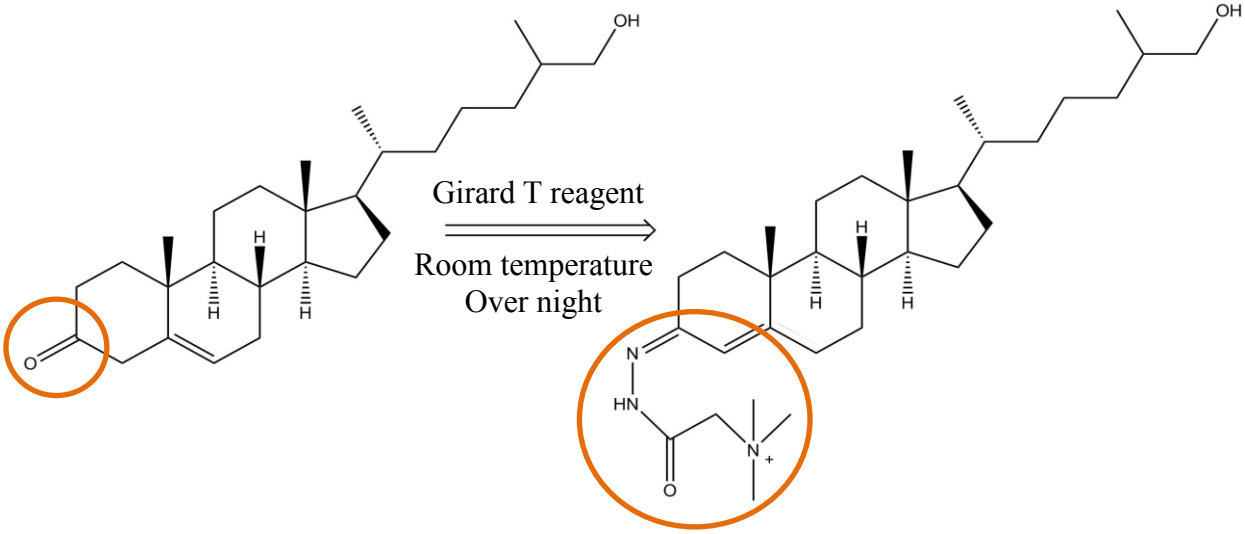


Figure 5: *27-OHC derivatized with Girard T reagent, illustrated with circle.*

Traditionally, the oxysterols have been analyzed by gas chromatography (GC) coupled to mass spectrometry (GC-MS) after derivatization to make the analytes volatile and thermally stable [45-47]. The sample preparation (including the derivatization step) can be time consuming and involves somewhat extra manual effort (compared to determination of oxysterols with LC) [39, 48-51]. In addition, some of the oxysterols might not necessarily be thermally stable at the

temperature required in GC mode [52, 53] and the sensitivity is not good enough compared with LC-ESI-MS [3]. Furthermore, when using GC, a hard ionization method is often used such as electron ionization (EI). EI operates traditionally at 70 eV, which yields extensive fragmentations of biological molecules. Identification of the molecular ion in the mass spectra and thereby determination of molecular mass can be tedious [42, 54, 55]. These are some of the reasons that methods where liquid chromatography with electrospray ionization coupled with mass spectrometry (LC-ESI-MS) have increased in popularity for the determination of oxysterols over the last years. Previously methods for determination of the oxysterols using LC-ESI-MS are not suited for screening application (*e.g.* 15 – 30 minutes analysis time [56, 57]) and which is why a rapid analysis is emphasized in this study (*e.g.* 5 minutes analysis time). LC-ESI-MS is described in further details in ***Section 1.6.***

1.3 Chromatography

In LC, a small sample is introduced to the inlet of a column which contains the stationary phase (SP) [58]. A mobile phase (MP) consisting of (typically) a pH adjusted mixture of water and organic modifier transports the compounds in the sample through the column. There, the compounds will have different interactions towards the SP and the MP [59, 60] illustrated in ***Figure 6***. Due to various interactions, the migration of the compounds will differ and they will elute from the column at different times [58, 61]. The time it takes for the analyte to pass through the column and be detected with a suitable detector is defined as the *retention time*, t_R. The retention of compounds in LC, especially in reversed phase LC (described in ***Section 1.3.1***) which has become the key separation principle for oxysterols [3, 50, 62], is a complex process that are difficult to describe exactly [58, 63-66]. However, expected interactions between the oxysterols and the hydrophobic SP (reversed phase LC) can be hydrophobic interactions [67], π-π interactions and dipole-dipole interactions [61, 68]

Elution of the analytes can be done isocratic, where the composition of the MP is constant, or by gradient elution where the composition of the MP is changed over time. Gradient elution is usually utilized when the sample is complex and the analytes have considerable diverse affinity towards the SP that results in early and late eluting molecules.

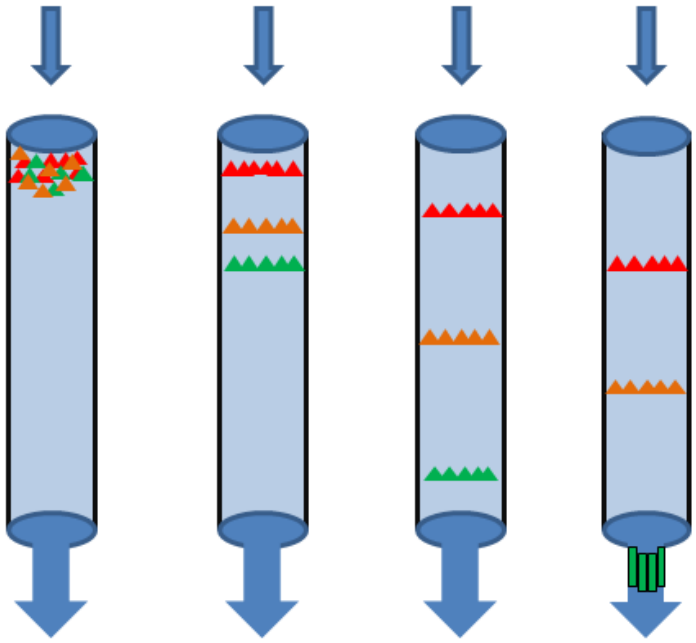


Figure 6: *An illustration of the separation of three different compounds (red, yellow and green) in a column. The compounds will elute from the column with different retention times depending on their interactions with the SP. Here, green has the shortest retention time (less interactions) while red has the longest (most interactions).*

1.3.1 Reversed phase liquid chromatography

Reversed phase liquid chromatography (RPLC) is the most common method for separating compounds in LC [61, 69, 70]. In contrast to normal phase liquid chromatography (NPLC), where the SP is polar, the SP in RPLC will be opposite or "reversed", hence a non-polar SP. NPLC has traditionally been the first choice for the separation of isomers [71], but are less compatible with ESI-MS because of the solvents typically used (*e.g.* poor ionization efficiency and sensitivity) [72]. In the review by Griffiths *et al.* [3] they describe several successful methods for separation of the hydrophobic oxysterols using RP-LC. The same approach will be used in this thesis where the SP is non-polar and based on silica particles with hydrophobic groups attached, where C_8 and C_{18} are the most commonly used as illustrated in ***Figure 7.*** The properties of the column particles are described in further detail in ***Section 1.4.2.*** The solvent or MP is polar and consists of water or aqueous buffer with a water-soluble organic solvent. This will lead to interactions (*e.g.* hydrophobic interactions) between the hydrophobic molecules and the SP, and hydrophilic molecules will pass through the column and be eluted

first. In general, a decrease in the polarity of the MP, equivalent to increasing the volume fraction of organic solvent in the MP, leads to a decrease in retention in RPLC mode.

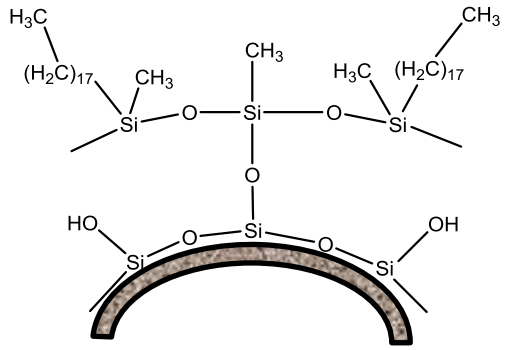


***Figure 7**: A SP consisting of polymeric silica-based materials with C_{18}.*

Different types of phases attached to the silica based surface were used in this study to separate the analytes. Different sorts of SP can provide different types of selectivity, and the phases investigated in this study are described in further detail in ***Section 1.5***.

1.4 Chromatographic performance

There are different ways to describe how well a chromatographic system performs. In this thesis, the chromatographic performance has been evaluated with regards to retention factor, resolution, column efficiency and peak shape as described below.

1.4.1 Band broadening

The band (a sample injected on a column) will broaden as it moves through the column, and which is the case in all chromatographic systems [58, 61]. One of the main reasons for this is the dispersion of the solute due to different concentration regions caused by the random movement of molecules (longitudinal dispersion) [73, 74]. Other contributions to band broadening inside the column are eddy dispersion and the resistance to mass transfer (both in the MP, the SP and the stagnant MP) [75]. When a column is packed with particles, it will be

challenging (or even impossible) to avoid gaps in between the particles, resulting in channels with different width and length [76, 77]. A band that is moving through and next to these channels will gain different velocity and give contribution to eddy dispersion. The velocity of the band is higher in the open channels and lower close to the particles. The resistance to mass transfer expresses the band broadening caused by transporting the analytes by dispersion and convection from one phase to the other.

Retention factor

At any given time during the migration through the system, there is a distribution of molecules of each component between the two phases (*i.e.* the SP and the MP). The retention factor is defined as the ratio of the amount an analyte in the SP to the amount in the MP. This ratio can be found experimentally by ***Equation 1***:

Equation 1 $$k = (t_R - t_M)/t_M$$

where t_R is the retention time for an analyte with interactions towards the SP and t_M is the migration time for a compound that has no interactions towards the SP.

If the sample is complex and contains several different compounds, some of them might co-elute (elute from the column at the same time). In this study the analytes are isomeric, and which means they have the same mass but different isomeric structure, thus, they will have the same signal in the MS. Consequently, the demands of the chromatographic separation (*e.g.* different k values) will be of high priority.

Column efficiency

The extent of band broadening determines the chromatographic efficiency, and is expressed as the plate number, N. The column efficiency (when using isocratic elution) is calculated using ***Equation 2***.

Equation 2 $$N = \left(\frac{t_R}{\sigma}\right)^2 \approx 5.54 \times \left(\frac{t_R}{w_{0.5}}\right)^2$$

where t_R is the retention time, σ is the standard deviation (assuming Gaussian distribution of the compound) and $w_{0.5}$ is the width of the peak measured at half height.

The plate height is another way to express band broadening, and is related to the width of the band emerging from the column. Lower plate height means sharper bands. The plate height is calculated by using ***Equation 3****:*

Equation 3 $$H = L/N$$

where L is the column length and N is the plate number. The terms plate number and plate height have their origin in the plate model of the chromatographic process [78, 79].

Higher flow rate reduce the contribution of longitudinal dispersion while smaller particles and core-shell particles (***Section 1.4.2***) reduce the band broadening contribution regarding both the resistance to mass transfer and eddy dispersion [58, 60, 80].

The column efficiency can be up to 200 000 plates m^{-1} depending on how well the column is packed, the size of the particles as well as what type of particles is being used (*e.g.* totally porous or core-shell particles) [58]. The calculation of the theoretical column efficiency can only be done with an isocratic elution. Also, larger analytes results in reduced column efficiency due to lower dispersion rates compared to smaller analytes.

The column efficiency for determination of oxysterols in previous works have been varying, where most articles just presented the column efficiency as "good" or "improved" without reveling what they compared it to [39, 81]. Griffiths *et al*. [62] separated 24S- and 27-OHC with an RP column using LC with atmospheric pressure chemical ionization and MS where the

retention time for 24S- and 27-OHC was 11.4 minutes and 12.8 minutes. Both peak width at half height was ≈ 1 minutes, giving a modest column efficiency of N ≈ 800.

Resolution

The separation of two peaks in a chromatogram can be described by their resolutions, R_S, and there are two factors that play a role in how well compounds are separated; the elution time between the peaks, t_{R1} and t_{R2} (**Δt_R**) and the average width of the peaks at half height, $w_{0.5\,(1)}$ and $w_{0.5\,(2)}$ (**$w_{0.5}$**). Narrow peaks with a gap between them give high resolution. There are different ways to calculate the resolution, but in this thesis, ***Equation 4*** is used [61], where the width of the peak is measured at half height of the peak (instead of measuring the peak at the baseline).

Equation 4

$$R_S = \frac{0.589\Delta t_R}{\frac{1}{2}w_{0.5}}$$

Previous works have provided sufficient resolution of the oxysterols (R_S ≈ 1-1.3) but with analysis time of > 20 minutes [82, 83]. McDonald *et al*. [84] did not manage to get acceptable resolution (*i.e.* for quantitation) between 24S- and 25-OHC (two closely eluting compounds) in LC-MS mode and concluded that the best alternative was to use GC-MS. R_S ≈ 1.5 is highly desirable for quantitative analyses.

Equation 4 is derived from ***Equation 5***, which can only be applied for closely eluting compounds.

Equation 5

$$R_S = \frac{1}{4}(\alpha - 1)\sqrt{N}\frac{k}{(1+k)}$$

The resolution of two peaks can be improved by increasing N (plate number), k (retention factor) or α (relative retention). α (also called the separation factor) is defined by the ratio of the relative retention factor (k_2/k_1) of two compounds.

Peak shape

In an ideal world, chromatographic peaks would always be Gaussian shaped but this is rarely the outcome. Peak asymmetry or tailed peaks can arise from several sources, both instrumental (*e.g.* column, capillary, tubing, detector *etc*) and chromatographic (*e.g.* longitudinal dispersion, eddy dispersion, resistance to mass transfer) [61, 71]. Furthermore, incomplete resolution of analytes, analyte interactions with uncoated sites on the SP, different chemical reactions and the formation of column voids can also effect band broadening [85, 86].

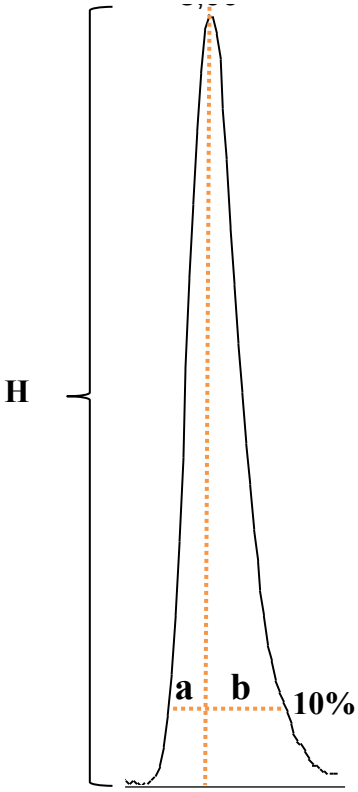


Figure 8: *Measurement of peak asymmetry. H is the peak height while a and b are measured at 10% of H.*

Column voids formed by bed shrinkage are usually a gradual process that evolves over time and causes progressive peak broadening. Voids in parts of the cross section along the length of the column can produce peaks with tailing (wider back half compared to the front half of a peak) or fronting (wider front half compared to the back half of a peak). Such peak distortions (tailing or fronting) decrease resolution, can interfere with quantitation and analytes with low concentrations can be lost in the tail of a major peak.

There are different ways to calculate asymmetric peak shape, but in this thesis, the asymmetry factor (A_S) is calculated at 10% of the peak height **(H)** where $A_S = b/a$ as illustrated in ***Figure 8***. A_S is expected to be as close to 1 as possible.

1.4.2 Core-shell particles

Traditionally, totally porous particles (with surface area in the 200-300 m^2/g range [71]) have been used for determination of oxysterols [3]. They are porous throughout the whole particle. Core-shell particles (also called solid-core, porous-shell, fused-core particles *etc*.), shown in ***Figure 9***, were first reported in the 1960s [87]. Core-shell particles have a solid core with a thin porous layer (in the range of 0.2 – 0.7 µm) on the outside. Core-shell particles have the

advantage that analyte mass transfer from the MP into and out of the outer porous layer of the particles is faster than in totally porous particles (diffusion of analytes in a thin porous layer instead of a whole particle). This provides a higher separation efficiency [88] (***Equation 2)*** due to less band broadening. As diffusion of analytes takes place in a thin layer on the core-shell particles, it is possible to apply larger core-shell particles which will provide the same efficiency as smaller totally porous particles [89, 90]. Hence, a higher flow rate can be utilized (with equal backpressure to totally porous particles) and which results in faster analyses [89, 91, 92]. However, the optimum flow rate can be restricted as described by the van Deemter equation (***Equation 6***) [58]. Since the c-term in the van Deemter equation is more flat for core-shell particles compared to totally porous particles (with the same particle diameter), a higher flow rate can be used for core-shell particles and still maintain the same column efficiency [93].

Equation 6 $$H = A + B/u + Cu$$

where A is eddy dispersion, B is the longitudinal dispersion in the MP, C is the resistance to mass transfer in the MP and the SP and u is the linear flow rate.

Totally porous particles have been the preference in analytical columns regarding the separation of oxysterol [3], but previous work by McDonald *et al.* [39] has shown improved chromatographic performance (*i.e.* rapid analysis with $R_S \approx 1.3$) when using core-shell particles instead.

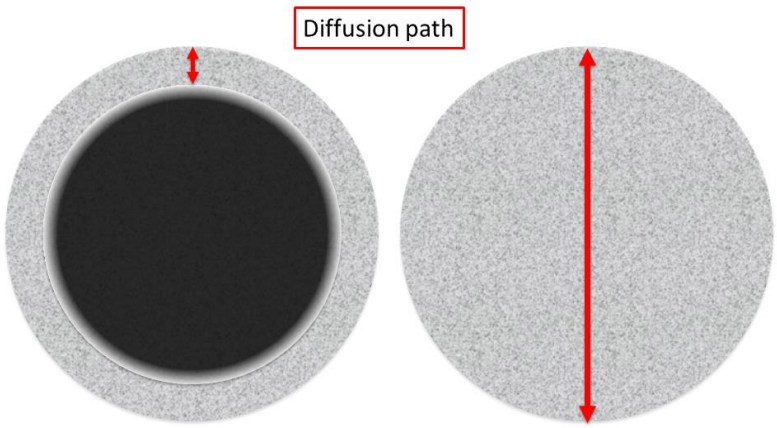


Figure 9: *The red arrow illustrates the diffusion path for mass transfer from the MP into and out of the outer porous layer of the particle and is shorter for core-shell particles (left) compared with totally porous particles (right).*

1.4.3 Adjustable parameters and conditions

The retention time of a compound can be altered depending the type of SP and MP that is used. Additionally, the flow rate (µL/min) of the MP, types of organic solvent, the ratio between organic solvent and water and the column temperature are different conditions that can be adjusted to control when a compound will elute.

Flow rate, pressure and particle size

Increased flow rate (with a MP that initially is capable of eluting the compound) will make the compound travel faster through the column and thereby decreasing retention time. The range of the flow rate is usually restricted by pressure and depends on the capacity of the pump, the connections and the couplings in the system and on the column itself (length, inner diameter, particles, SP *etc*).

The particle size of packing materials in conventional columns is typically 2 to 5 µm, while the ultrahigh-pressure system employ sub-2 µm particles. The backpressure resulting from pumping the MP through the column is given by:

Equation 7

$$P = \frac{\kappa u \eta L}{d_p^2}$$

κ is a constant, u is the linear flow rate, η is the viscosity ($kg*m^{-1}*s^{-1}$), L is the column length and d_p is the particle diameter.

Thus, the backpressure is doubled by doubling the column length. When the particle size is reduced from 5 to 3 µm, the pressure increase almost three times with the same column length.

The smaller the particles in the packing material the higher the backpressure will be. Thus a system that can deliver a higher pressure to the MP is required, and the term high pressure liquid chromatography (HPLC), or more common high performance liquid chromatography, was introduced [61]. The main parts of the HPLC consist of a pump, an injector, a column and a type of data handling device as shown in ***Figure 10***.

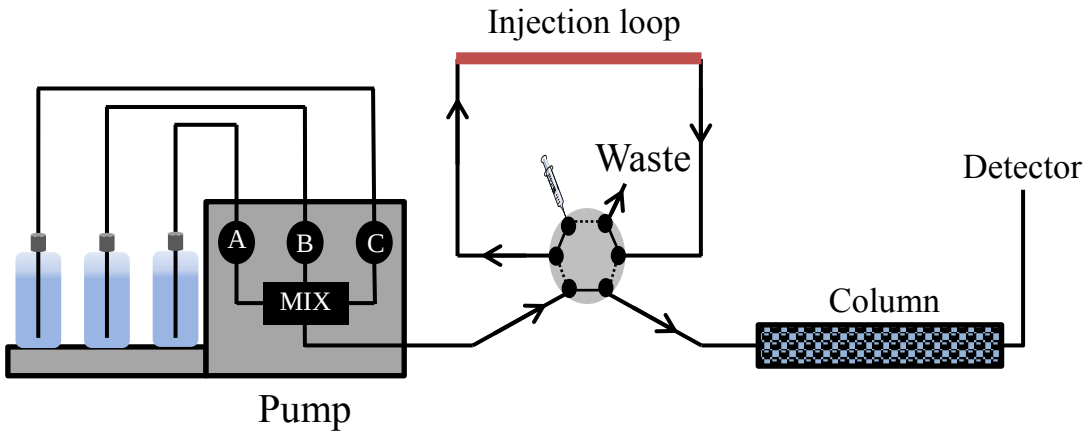


Figure 10: *An HPLC system, here illustrated with a pump with three channels for different solvents, a 2-way-6-port valve for injection (here illustrated with manual injection) and a column where the separation is done. After the separation the compounds will be detected in a detector.*

Temperature

The retention in RPLC is temperature dependent. Increasing temperature reduces the retention for most compounds [58]. Asymmetric peak shapes are also often improved by increasing the column temperature [58]. Improved peak shapes at higher temperatures are often related to the fact that the diffusion coefficient D increases with increasing temperature, as shown by the Stokes-Einstein equation (***Equation 8***), improving the mass transfer in both phases. The viscosity decreases at the same time:

Equation 8 $$D = RT/6\pi\eta N'r$$

η is the viscosity, N' is the Avogadro number, r is the molecular radius, T is the temperature and R is the gas constant.

Since elevated temperatures reduce the viscosity of the MP and thereby reduce the backpressure, higher speed and improved resolution can often be obtained by selecting a column temperature above room temperature. Usually, separation of oxysterols have been performed with the analytical column at room temperature or at slightly elevated temperatures (20 - 40 °C) [39, 52], and which may indicate that the effect of adjusting the column temperature has not yet been fully explored.

Organic solvent and the ratio to water

The retention for a compound in RPLC mode with a specific MP mixture (organic solvent and water) can roughly be described by ***Equation 9****:*

Equation 9 $$log\, k = log\, k_W - S\Phi$$

k is the compound retention factor, k_W is the compound retention factor for water as MP (water has the weakest elution strength in RP), Φ is the volume fraction of the organic solvent and S is the slope of experimental data after fitting it to a linear regression model [71]. Supposing that the composition and the volume of the SP are not affected by the change in composition of the MP, an estimate can be used to define the S-value as a measure of the solvent strength of the organic solvent. S-values are widely used in method development to select a MP composition of the same strength but different selectivity [94]. S-values do vary, so it is only considered as a guiding tool. Selected S-values are given in ***Table 3*** and are based on Snyder's selectivity triangle [95].

Table 3: *The solvent strength and selectivity parameters based on Snyder's selectivity triangle. (S is an empirical solvent strength parameter for RP-LC). Values from [71].*

Solvent	**Solvent strength**		**Solvent selectivity***		
	P'	**S**	**x_e**	**x_d**	**x_n**
Ethanol	4.3	3.6	0.52	0.19	0.29
Acetonitrile	5.8	3.1	0.31	0.27	0.42
Methanol	5.1	3.0	0.48	0.22	0.31
Water	10.2	0	0.37	0.37	0.25

**The sum of the three polar distribution constants provides a measure of the solvent strength (P') and the ratio of the individual polar distribution constants to their sum a measure of selectivity (x_n, x_e and x_d). x_n, x_e and x_d is solvent capacity for dipole-type, hydrogen-bond base and hydrogen-bond acid interactions, respectively.*

As shown in ***Table 3***, the solvent strength in RPLC is increasing when changing the organic solvent from methanol to acetonitrile, and which means that acetonitrile will elute hydrophobic

analytes faster than methanol would. Mixtures of organic solvents (*e.g.* mixture of acetonitrile and methanol) can provide different selectivity (*e.g.* different retention factors that can improve resolution between the analytes) and have been used for determination of oxysterols before [39, 44, 51, 88] but has not yet been fully explored.

1.4.4 Column dimensions

Today, the inner diameter (ID) of conventional liquid chromatography (LC) columns are in the range of 2 - 5 mm. Capillary columns typically have an ID of > 0.1 – 0.5 mm while nano columns have ID <0.1 mm, as given in ***Table 4*** [96]. Smaller ID has several advantages such as less radial dilution that in turn provides more concentrated chromatographic bands eluting from the column. They also requires a lower flow-rate, hence they use less solvent compared to those of larger ID. Smaller ID of the columns will improve the detection limit with concentration sensitive detectors such as ESI-MS [58] when injecting the same sample amount. This is due to the reduced radial dilution of the chromatographic bands eluting from the column (compared to a column with larger ID) [97]. This principle is illustrated in ***Figure 11***. The enhanced sensitivity due to this can be described by the downscaling factor (DF), ***Equation 10***.

Equation 10

$$DF = \frac{[Inner\ diameter\ of\ column\ 1]^2}{[Inner\ diameter\ of\ column\ 2]^2}$$

The radial dilution is proportional with the square of the radius of the column. By downscaling from 2.1 mm to 0.1 mm, a 440 times more concentrated band will elute from the column.

Several column dimensions (*e.g.* 0.3 mm and 0.1 mm ID) have been explored for separation of oxysterols in the past few years [56, 57]. The sensitivity has been good (*e.g.* detection in low ng/mL range), but there are still work to be done if the determination of oxysterols should be put in a diagnostic screening setting where analysis time should not exceed ≈ 10 minutes and still maintain acceptable resolution for quantitative determination.

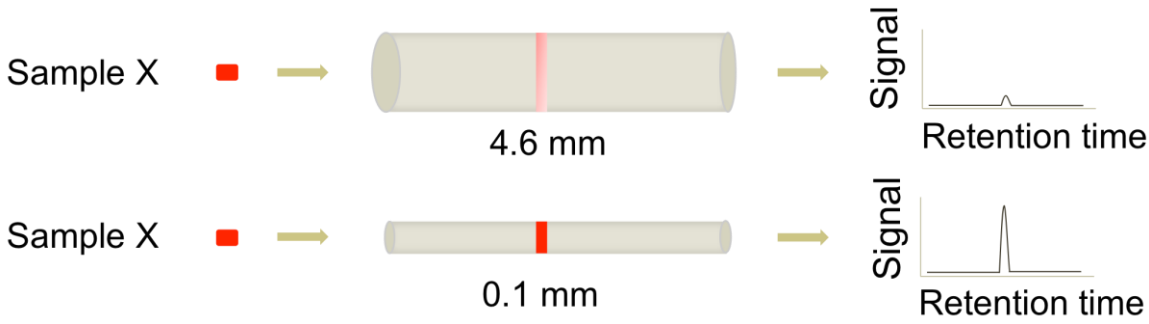


Figure 11: *Radial dilution is a function of the square of the column radius. Therefore, when using a narrow ID, the compounds eluting from the column will enter the ESI-MS with a more concentrated band resulting in an enhanced signal. Adapted from [98].*

Table 4: *Column dimensions in HPLC* [96].

Column type	ID (mm)	L (cm)	d_p (µm)	Typical flow µL min^{-1}
Conventional	2-5	3-25	2-5	100-2000
Microbore	0.5-1	3-25	2-5	20-100
Capillary	0.1-0.5	3-25	2-5	1-20
Nano	0.01-0-1	5-25	2-5	0.02-0.2

1.5 Stationary phase

Silica is undoubtedly the most important support used in RPLC due to its versatility and physical characteristics [63, 99]. The silica functions as a substrate where different materials can be chemically bonded to it, thus producing a wide range of selectivity for different analytes. Unreacted hydroxyl groups attached on the silica surface can introduce hydrogen bonding interactions as an additional interaction mechanism (termed, secondary interaction) [100]. This can provide unwanted retention of the compound, resulting in poor repeatability of retention times and asymmetrical peak shapes [101]. Several column manufactures uses endcapping technology resulting in fewer (unwanted) unbound silanol groups. Analytical columns with C18 SP are the most popular choice in the scientific community for separation of oxysterols [56, 72, 84, 102] and are illustrated in ***Figure 12***, where the Hypersil Gold C18 is one of the favorites [44, 52, 103-105]. Separation of oxysterols with phenyl hexyl SP have resulted in rapid analysis time in previous studies [106] and the SP is illustrated in ***Figure 13***.

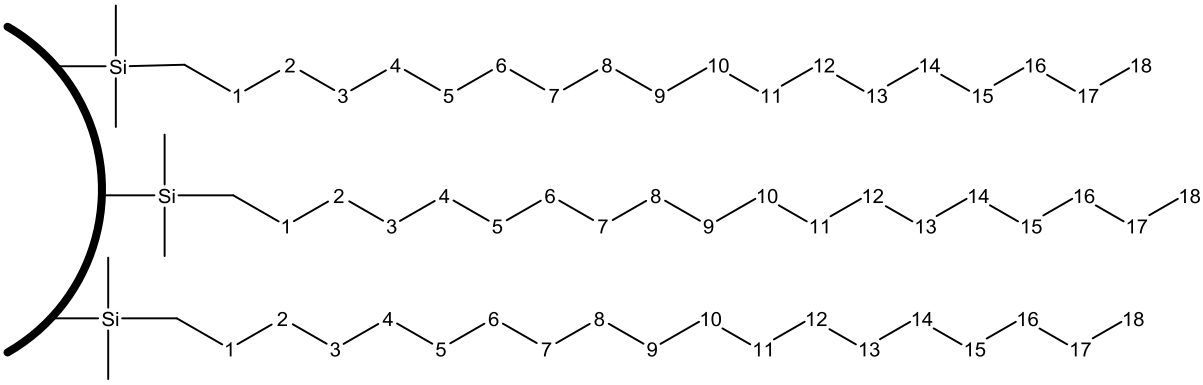


Figure 12: *An illustration of the SP where a C_{18} alkane chain is attached to the silica surface.*

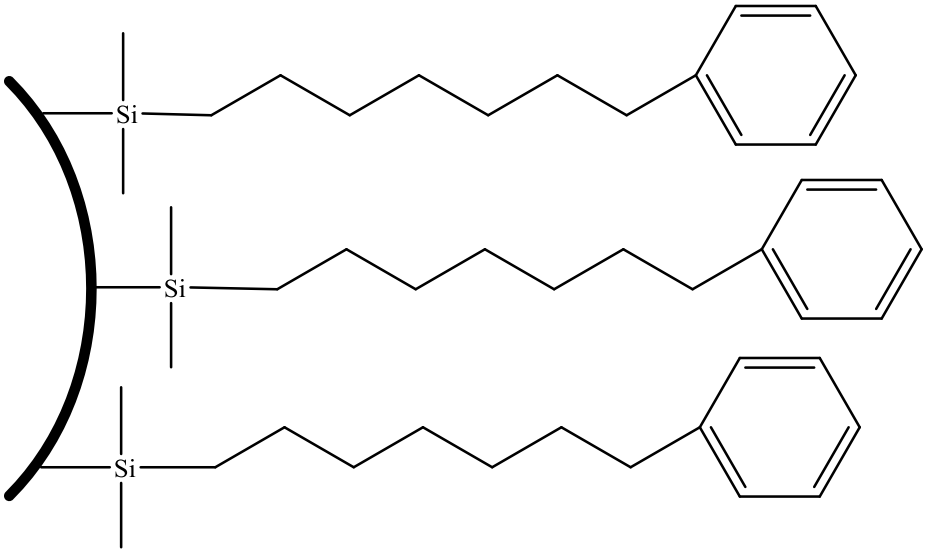


Figure 13: *An illustration of a phenyl hexyl SP where a phenyl group is attached to the particles by a straight alkane chain.*

1.6 Electrospray ionization mass spectrometry

The LC-MS is a major tool in analyses of biological samples because of its high sensitivity and selectivity. One of the challenges is that the LC operates with liquids at atmospheric pressure and the MS instrument operates under high vacuum. A proper interface between the LC and MS detector is therefore essential. The most common interface in LC-MS is the ESI. The ESI is operated at atmospheric pressure and is appropriate for charged and ionizable compounds. It is a soft ionization technique which gives less fragmentation compared to EI [58, 107]. The ESI process is illustrated in ***Figure 14.*** The MP containing the analytes enters a capillary where a high voltage is applied. When using a positive voltage, cations will migrate with the MP to the outlet of the capillary. Due to repulsion between the positively charged capillary and the repulsion between the cations, an abundance of cations accumulates at the outlet of the capillary. At the outlet of the capillary they will form a so-called Taylor cone and which subsequently will explode into droplets that contain a surplus of positively charged particles. Due to repulsive forces inside the droplets, they explode into smaller droplets as they move towards the entrance of the MS. This ultimately provides ions in gas phase (ejected from the droplets) which enter the MS [108].

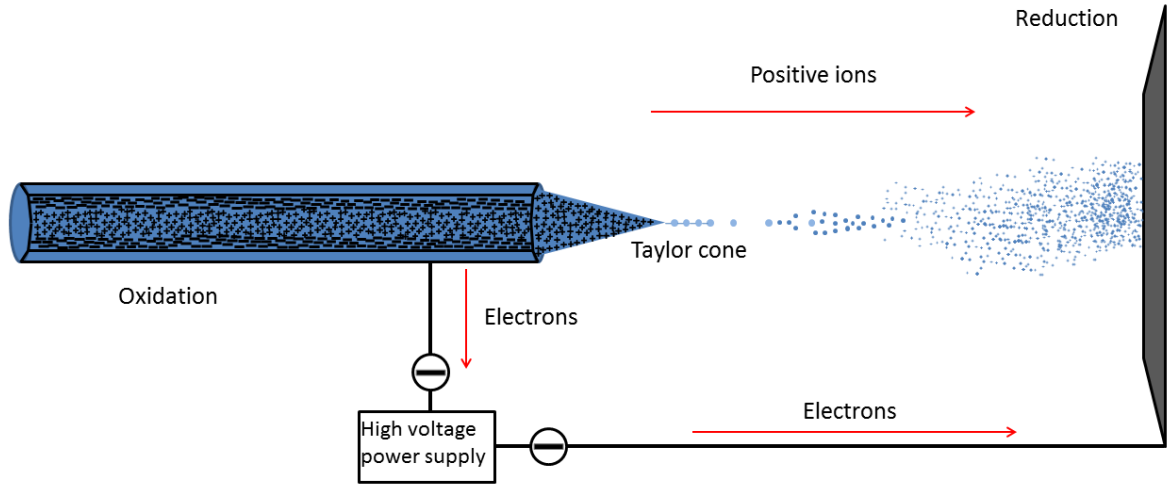


Figure 14: *The principle of ESI. When high voltage is applied, ions will follow the MP and move towards the capillary outlet. Due to repulsion, the cations migrates to the outlet of the capillary where they form a Taylor cone. Repulsive forces will break the cone into small, highly charged droplets. The droplets undergo fission and become even smaller highly charged droplets. Eventually ions in gas phase are produced. Here shown with positive ionization mode.*

Triple quadrupole mass spectrometer

After the analytes are ionized, they leave the ion source and enter the MS through a series of lenses and skimmers. The lenses and skimmers are focusing the ion beam leaving the ionization source in addition to dividing the MS into several compartments where the pressure is gradually reduced by vacuum pumps. It is essential to remove air (gas molecules) inside the MS by creating a vacuum so that ions entering the MS can *fly* towards the mass analyzer without bumping into other gas molecules. The mass analyzer inside the MS is where ions are separated according to their mass-to-charge ratio (*m/z*). There are several types of mass analyzers, but in this study, two different triple quadrupole mass spectrometers are used; the TSQ Vantage and the TSQ Quantiva, both from Thermo Fisher Scientific. The triple quadrupole mass spectrometer (TripleQ-MS) has three stages of quadrupoles, where each stage can be operated separately by applying altered electrical fields to them, illustrated in ***Figure 15.*** In each quadrupole stage, there are four rods placed parallel to each other (illustrated with only one rod for collision cell in ***Figure 15***) and where opposite pairs of rods are connected electrically. By applying both a certain direct current (DC) and a radio frequency (RF) on one of the pairs and the opposite DC and RF on the other pair, an oscillating electrical field is created. When ions enter the field in the z-direction (from the ion source), they start to oscillate in the x- and y-direction. When the ions have a stable trajectory, they will pass through the rods and be detected. Only specific mass to charge (*m/z*) values are able to pass the quadrupole when certain DC and RF values are applied.

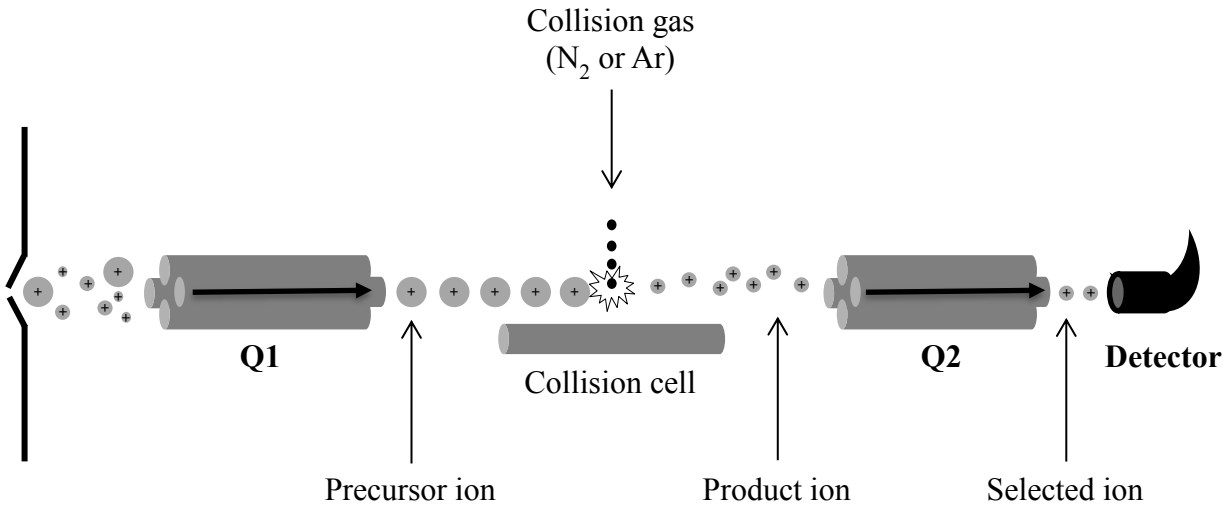


Figure 15: *An illustration of the TripleQ-MS. Q1 and Q2 are operated with specific DC and RF values to provide a stable trajectory for the investigated ions. Here illustrated with selected reaction monitoring mode.*

In the TripleQ-MS, two of the quadrupoles (the first and the last) are normally operated with different DC and RF values. The middle quadrupole is used as a collision cell filled with an inert gas and only RF is applied on the rods [109]. This is where fragments of the analyte ion are produced. This feature is termed tandem MS (MS/MS), and provides the possibility to monitor molecular ions passing through Q1 (***Figure 15***) as the parent ion entering the collision cell where fragment ions are formed and possibly detected if Q2 provides a stable trajectory (pre-selected *m/z* value) for the ion. The MS/MS feature enables the possibility to monitor the ionized analyte and fragments of it and which gives a characteristic fingerprint that is unique for the specific analyte. The fragments to be monitored have to be selected based on their intensity and by using computer software to verify that they are unique for the specific analyte. The different ways to operate the triple-MS are illustrated in the appendix in ***Figure S 3***.

1.7 On-line sample clean up

Oxysterols are neutral compounds that must be derivatized to charged species in order to be measured with high sensitivity by ESI-MS [82]. When using the derivatization reagent to charge the analytes, an excess of reagent is added to the sample. It is therefore necessary to remove the excess reagent before introducing the samples into the LC system to avoid overloading the column and also avoid precipitating of excess derivatization reagent in the LC system or column. If the column is overloaded, troubles with peak broadening might occur [110]. If the excess derivatization reagent reaches the detector in the MS instrument, it could be contaminated. The sample clean up procedure with solid phase extraction (SPE) (also called pre-column or trap column) is done off-line or on-line with an on-line 10-port valve system. The switching system (the 10-port valve) enables the possibility to inject a larger sample volume onto the column without risking overloading it and/or getting extensive band broadening [82]. Limited injection volume (sample amount) is one of the major drawbacks with narrow ID columns. The maximal injection volume on a 1.0 mm ID x 150 mm column with 5 µm particles should according to Vissers *et al*. [111] be 0.5 µL to avoid extra band broadening. By using a switching system with an on-line SPE column, it is possible to inject a larger sample volume (*e.g.* 100 µL) with a separate loading pump delivering a high flow of non-eluting MP where the SPE column consequently trap the analytes and enrich them.

A robust automatic filtration and filter back-flush with solid phase extraction (AFFL-SPE) system allows on-line sample clean up to avoid doing manual off-line SPE [56, 82, 112, 113]. The AFFL-SPE system is shown in ***Figure 16***. The sample that contains analytes, particles (*e.g.* cell debris, protein precipitant from biological samples) and excess Girard T reagent (a common derivatization reagent [57]) is loaded on the SPE column with a loading pump after the sample has passed through a stainless steel filter. Particles are confined on the filter and analytes are trapped on the SPE column (with a hydrophobic SP) while hydrophilic compounds are eluted to waste (including excess derivatization reagent). When the 10-port valve switches, the LC MP flushes the SPE column backwards (back-flushing) and transfers the analytes from the SPE column to the analytical column for separation. While the LC pump elutes the analytes to the column, the loading pump flushes through the filter backwards and transports particles to waste. The filter is then ready for the next sample.

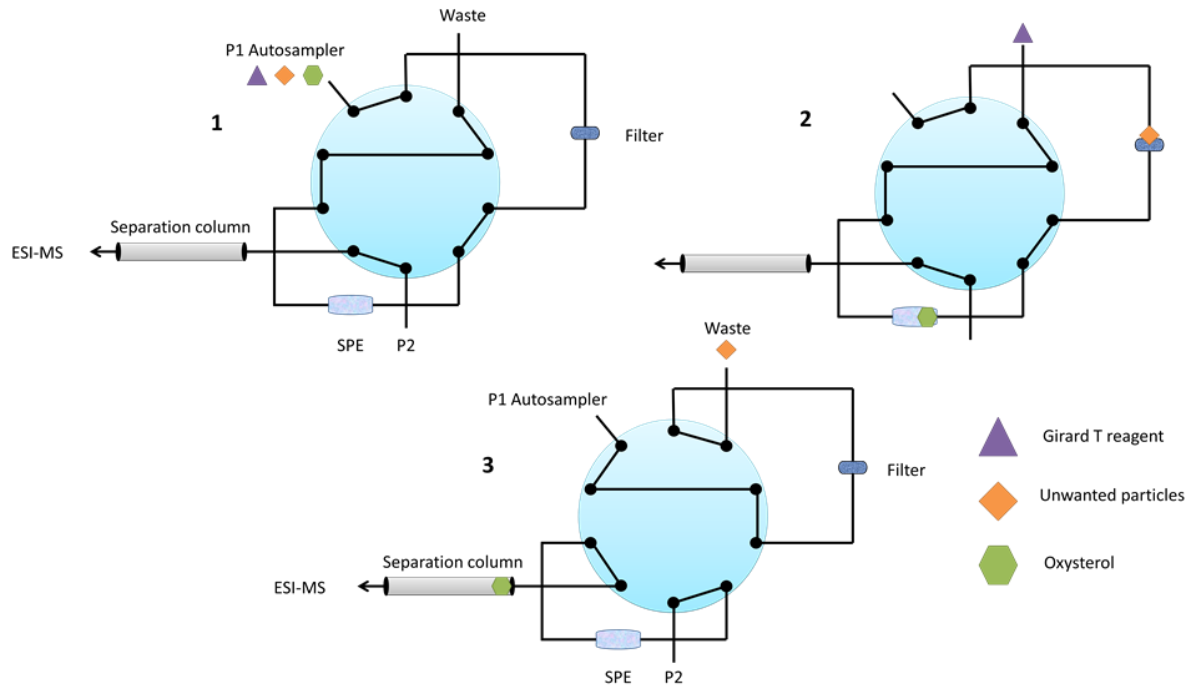


Figure 16: *The AFFL-SPE-LC system for determination of oxysterols (green) in samples containing unwanted particles (e.g. salts or cell debris) (orange) with the presence of excess derivatization reagent, Girard T (purple).* ***1)*** *The mixture is loaded.* ***2)*** *Unwanted particles are trapped on a stainless steel filter, the oxysterols are trapped on the SPE column and the excess reagent is flushed to waste.* ***3)*** *The oxysterols are eluted on to the analytical column while unwanted particles are back flushed off the stainless steel filter to waste.*

1.8 Aim of study

When determination of oxysterols is to be done in a diagnostic screening setting, the demand for a fast analysis (*e.g.* high throughput) with secure identification of each analyte (*i.e.* $R_S \approx 1.5$) is highly desirable. This will be addressed in this thesis by exploring the benefits from core-shell particles (*e.g.* less backpressure at high flow rates), elevated column temperatures (hence improved peak shape), narrow ID columns (less radial dilution *i.e.* increased sensitivity), mixtures of organic solvents and the use of large volume injection by implementing the AFFL-SPE system, as these are key parameters have yet to be fully explored.

A key goal was to develop an LC-MS approach that enables chromatographic separation ($R_S \geq 1.5$) of the side-chained hydroxycholesterols 22R-, 24S-, 25-, 27-, and 22S-OHC within 5 minutes. The method developed will be used for the determination of oxysterols in biological samples.

2 Experimental

2.1 Chemicals

HPLC gradient grade methanol (MeOH), LC-MS grade acetonitrile (ACN) and LC-MS grade ethanol (EtOH) were all purchased from VWR (Radnor, PE, US). LC-MS grade formic acid (FA), Girard T reagent, KH_2PO_4, 22S-OHC (cholest-5-ene-3β,22(S)-diol), 25-OHC (cholest-5-ene-3β,25-diol), 22R-OHC (cholest-5-ene-3β,22(R)-diol) and cholesterol oxidase from *Streptomyces sp*. were all bought from Sigma Aldrich (St. Louis, MO, US). 27-OHC (cholest-25(R)-5-ene-3β,26-diol and 24S-OHC (cholest-5-ene-3β,24(S)-diol) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Glacial acetic acid was obtained from Merck (Merck KGaA, Darmstadt, Germany). Type 1 water (18.2 MΩcm resistivity at 25 °C) was from a Milli-Q integral purification system dispenser (Millipore, Billerica, MA, US). 2-Propanol and chloroform were purchased from Rathburn Chemicals LTD (Walkerburn, Scotland, UK). Carbon tetrachloride was bought from Prolabo (Rectapur, acquired by VWR). "Frit kit" consisting of Kasil 1624 (potassium silicates/water (29/71 (*v/v*)) and formamide were bought from Next Advance (Averill Park, NY, US).

2.1.1 Items used for preparation of solutions and standards

The analytical fine mass balance was from Mettler-Toledo (Model AE166, Columbus, OH, US). All pipettes and tips were from Thermo Scientific (Waltham, MS, US). 100 mg C_{18} Isolute SPE columns (for off-line SPE) were from Biotage (Uppsala, Sweden) and 100 mg C_{18} Oasis SPE columns (for off-line SPE) were from Waters (Milford, MS, US).

2.1.2 Containers and laboratory glass ware

The glass vials (1.5 mL Chromatography Autosampler vial), the autosampler vials (0.3 mL Microvials) and caps (Snap Ring Cap (11 mm)) were all from VWR. The safe-lock tubes and Protein LoBind tubes (1.5 mL) were from Eppendorf (Hamburg, Germany). The glass flasks were from Schott (Mainz, Germany) and the measuring cylinders were from Kimble (Fisher scientific, part of Thermo Scientific).

2.1.3 Items used for packing columns

The plastic syringe was bought from BD Plastipak™ (1 mL Luer syringe, Becton Dickinson, Francling Lakes, NJ, US). PTFE sealing tape was purchased from Sigma Aldrich. The ultrasonic baths were from Branson (Branson Ultrasonics, Dansbury, US) with model number 5510 and 200, or ATU (Aplicaciones Téchnicas De Ultrasonidos, Paterna, Valencia, Spain, model number ATM50-0.7LC). The use of ultrasonic baths was based on availability. The 100 DM packing pump was from ISCO (Teledyne ISCO, Lincoln, NE, US). The packing chamber, a stainless steel tube (6.35 x 125 mm) with stainless steel tube fittings was from Swagelok (Solon, OH, US). The 17A gas chromatography oven was from Shimadzu (Kyoto, Japan).

2.1.4 Connections and items used for couplings

The polyether ether ketone (PEEK) tubings were purchased from IDEX Health and Science (Oak Harbor, WA, USA). The fused silica capillaries (FSC) were from Polymicro Technology (Phoenix, AZ, US). The Viper™ Capillary Stainless Steel Fingertight Fittings (Viper SSF), the nanoViper™ Fingertight Fittings (nanoViper) and the grounding stainless steel 1/16" union (grounding union) were purchased from Thermo Scientific. The Upchurch PEEK Fingertight nut with Fingertight ferrule (both 1.6 mm), the Upchurch PEEK Microtight® Connector Butt with MicroFingertight I Fittings and the 360 µm ID Upchurch Microtight® Tubing Sleeve were all purchased from Sigma Aldrich. The column filters (1/16" OD, 1 µm), the ZU1C stainless steel unions (ZU1C union, 1/16" ID), the fused silica FS1.4 ferrules (Vespel/graphite) and the steel nuts (1.4 mm and 1.6 mm) were bought from Vici Valco (Houston, TX, US).

2.1.5 Other equipment

The centrifugal evaporator (Concentrator Plus) and the centrifuge (model 5424R) were obtained from Eppendorf. The vacuum pump for centrifugal evaporator was from Edwards (model: E2M2, Crawley, Sussex, England). The direct infusion syringe (500 µL) was purchased from Thermo Scientific. The vortex MS2 MiniShaker was from IKA (Staufen, Germany) and the thermoshaker was from Grant-Bio (model: PHMT, PSC-20, 20x2.0 mL, Grant instruments, Shepreth, Cambridgeshire). The Titrino plus 877 pH meter was from Metrohm (Herisau, Switzerland). The nebulizing gas/fragmentation gas of argon (≥99.999%) was purchased from Praxair (Oslo, Norway). The column oven was from Spark Mistral (Spark Holland, Emmen,

The Netherlands) model number 880. The microscope (W10x/20) was from Motic (Hong Kong, China).The loading pump was from Hitachi (Chiyoda, Tokyo, Japan, model no. L-7100). The 2-way-4-port Cheminert manual injector was from Vici Valco (product no. C4-1004-.05)

2.2 Standards and solutions

2.2.1 Preparation of solutions

The MP was made by mixing (either by measuring separately with measuring cylinder or by using the LC pump) type 1 H_2O with MeOH, EtOH or ACN, all with 0.1% FA. A stock solution of 50 mM pH 7 phosphate buffer with cholesterol oxidase was prepared by dissolving 885 mg KH_2PO_4 in 130 mL type 1 H_2O with 4 mg cholesterol oxidase from *Streptomyces sp.*. pH adjustment to pH 7 was done by adding 1M NaOH solution dropwise while monitoring pH constantly with a pH meter. Derivatization solution was made by mixing 15 µL glacial acetic acid, 15 mg Girard T reagent and 500 µL MeOH per standard solution.

2.2.2 Preparation of stock solutions and working solutions for method development

The sample preparation, including oxidation with cholesterol oxidase and derivatization with Girard T reagent, is the same as described by Røberg-Larsen *et al*. [57, 82] and are based on the procedure described by Griffiths and co-workers [42]. The stock solutions of hydroxycholesterol were prepared by dissolving obtained standards of hydroxycholesterol in an appropriate amount of 2-propanol. The concentrations for each prepared stock solution is given in ***Table 5.***

Table 5: *Concentrations for each stock solution of hydroxycholesterol.*

Compound	Stock solution µg/mL in 2-propanol
22R-OHC	100
24S-OHC	100
25-OHC	188
27-OHC	500
22S-OHC	60

The preparation of hydroxycholesterol with oxidation and derivatization in a working solution was performed according to previously published procedure with minor modifications [57, 82, 114]. See the appendix ***Section 6.1*** for more details.

2.2.3 Preparation of biological samples

The preparation of plasma samples is described in the appendix ***Section 6.1.3*** and the preparation of tumor sample is described in the appendix ***Section 6.1.4***.

2.2.4 Off-line sample clean-up of standard solutions for direct infusion and method development

To remove excess Girard T reagent, an Isolute SPE column was used. The off-line sample clean-up is shown schematically in ***Figure 17*** and are based on a "recycling" method by Shoda *et al*. [115] which again was modified by Karu *et al*. [116]**.** The SPE column was conditioned with 1 mL MeOH followed by 1 mL type 1 H_2O and then with 2 mL MeOH / type 1 H_2O (1/1, *v/v*). The derivatized standard solution (720 µL in ~70% MeOH) was loaded on the conditioned SPE column and was eluted with approximately 1 drop every second to a 5 mL glass vial. The eluate in the glass vial was diluted with 2 mL type 1 H_2O and loaded onto the SPE column again where the eluate this time was eluted to waste. To wash out polar compounds and excess derivatization reagent, 2 mL of type 1 H_2O was added to the SPE column. The derivatized analytes that were trapped on the SPE column was eluted with 1 mL MeOH and then 1 mL chloroform. This solution was evaporated into dryness and re-dissolved in 700 µL 70% MeOH.

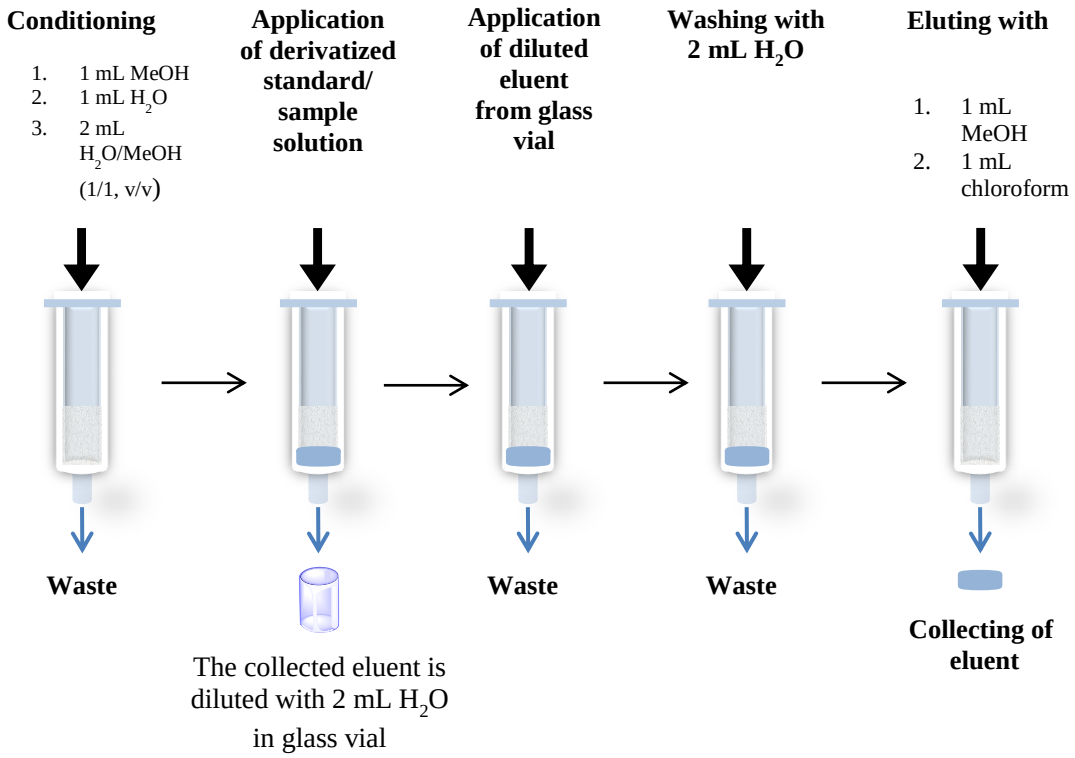


Figure 17: *A schematic description of the off-line sample clean-up procedure.*

2.2.5 On-line sample clean-up for standards and samples

The AFFL-SPE system for on-line sample clean-up was the same as described in the introduction in ***Section 1.7***. The loading pump used for loading the sample onto the SPE column delivered a non-eluting MP consisting of H_2O with 0.1% FA.

2.3 Columns and stationary phases

The different columns investigated in this study are given in ***Table 6*** with the properties for the particles in ***Table 7***.

Table 6: *Overview of the different columns with SPs, batch/lot no, dimensions and suggested interaction.*

Name/materials	**Batch / Lot no**	**Dimensions**	**Suggested interactions**
ACE UltraCore 2.5 SuperPhenylHexyl	V15-8818 Serial no: A174508 and Serial no: A183921	150 x 2.1 mm ID, 2.5 µm core-shell particles	$\pi - \pi$ interaction, dipole-dipole and hydrophobic interaction.
ACE UltraCore 2.5 SuperPhenylHexyl	V15-8818 Serial no: A198694	150 x **0.5** mm ID 2.5 µm core-shell particles	$\pi - \pi$ interaction, dipole-dipole and hydrophobic interaction.
Waters ACQUITY UPC^2 Torus 2-PIC	0104360951	100 x 2.1 mm ID, 1.7µm particle	$\pi - \pi$ interaction.
Waters ACQUITY UPC^2 Torus 1-AA	0105361261	100 x 2.1 mm ID, 1.7µm particles	$\pi - \pi$ interaction.
Thermo Hypersil GOLD C_{18}	13383	50 x 1.0 mm ID, 1.9 µm particles	Hydrophobic interactions.
ACE UltraCore 2.5 Super C_{18}	V17-1073	150 x 2.1 mm ID, 2.5 µm core-shell particles	Hydrophobic interactions.
Kromasil C8-100 5 µm SPE column	DT 0292	5.0 x 1.0 mm ID, 5 µm particles	Hydrophobic interactions.
HotSep Tracy Kromasil C4 300Å SPE column	-	5.0 x 1.0 mm ID 5 µm particles	Hydrophobic interactions.

Table 7: *The properties for the particles in the investigated columns [117-119].*

Name/materials	Functional group	Particle size (µm)	Pore size (Å)	Surface area (m^2/g)	Carbon load (%)
ACE UltraCore 2.5 SuperC18	Octadecyl encapsulated	2.5	95	130	7.0
ACE UltraCore 2.5 SuperPhenylHexyl	Phenyl-Hexyl encapsulated	2.5	95	130	4.6
Thermo Hypersil GOLD C_{18}	Octadecyl	1.9	175	220	11
Waters ACQUITY UPC² Torus 2-PIC	2-picolylamine endcapped	1.7	130	185	-
Waters ACQUITY UPC² Torus 1-AA	1-amino-anthracene endcapped	1.7	130	185	-

2.4 Column packing procedure

Emptying the 2.1 mm ID ACE SPH column

All in-house packed columns in this thesis have been packed with particles from the 2.1 mm ID ACE SPH column with bach no: V15-8818 and serial no: A174508. Before the packing could start, the commercial column (the 2.1 mm ID ACE SPH column) had to be emptied. The filter on the outlet of the column was removed and the column was connected to a pump (the Dionex UltiMate 3000 HPLC pump, ***Section 2.5.1***). The column was then pressurized with 0.2 – 0.4 mL/min pure MeOH. The SP was flushed out of the column and was collected in a safe-lock tube and dried in an oven for 20 min at 70 °C.

2.4.1 In-house packing of microbore and capillary columns

Preparation of the slurry for 0.3 mm x 150 mm column

An aliquot of 30 (± 1) mg of the dried SP (from the ACE SPH column) was mixed with 200 µL carbon tetrachloride in a 5 mL glass vial to make a slurry. The slurry was sonicated in an ultrasonic bath for 10 minutes before it was transferred to the packing chamber.

Preparation of the slurry for 0.5 mm x 150 mm column

An aliquot of 85 (± 1) mg of the dried SP (from the ACE SPH SP) was mixed with 560 µL carbon tetrachloride in a 5 mL glass vial. The slurry was sonicated in an ultrasonic bath for 10 minutes before it was transferred to the packing chamber.

The column packing set-up and the packing procedure

The Omniflex 1 mL plastic syringe used to transfer the slurry to the packing chamber was constructed as shown in ***Figure S 4*** in the appendix. The fused silica capillary (320 µm ID) had a length of approximately 12 cm.

At one end of a stainless steel column body (empty and clean) with the desired dimensions (0.5 or 0.3 mm ID x 150 mm) a ZU1C union with a graphite ferrule and a filter was mounted. The packing chamber was connected to the other end of the column (this end is without filter) as illustrated in ***Figure 18***. The sonicated slurry was transferred into the packing chamber using the constructed syringe. While transferring the slurry, the syringe was pulled slowly out of the chamber to avoid getting air into the slurry. The packing chamber was connected to the packing pump and the column was packed by increasing the pressure from 0 bar to 650 bar in 6.5 minutes with a packing liquid consisting of ACN/H_2O 70/30 (*v/v*). The pressure was kept at 650 bars for 15 minutes. After 15 minutes, the pressure was reduced to 0 bar and the column was disconnected from the packing chamber. A union with a filter was mounted on the end of the column which had been connected to the packing chamber. The column was coupled to the Dionex UltiMate 3000 HPLC pump where a MP consisting of MeOH with 0.1% FA was delivered at a low flow rate (≈ 10 µL/min) for 10 minutes, allowing the particles to settle before the column was used.

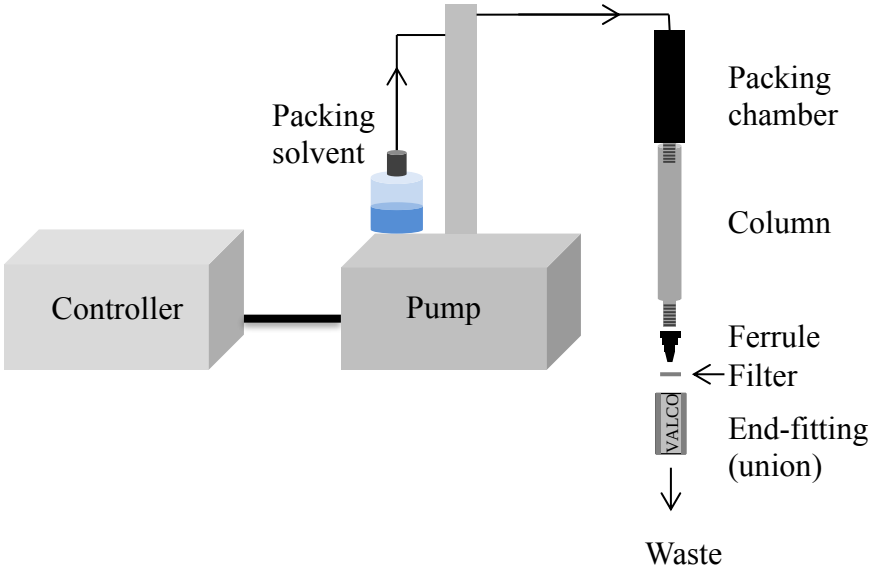


Figure 18: *An illustration of the packing system used for packing 0.5 mm and 0.3 mm columns.*

2.4.2 In-house packing of nano columns

The packing procedure for the nano columns were based on the standard operation procedure as described by H. S. Berg in her master thesis [120].

A silica capillary with the desired ID was cut in the desired length, including approximately 5-10 cm to make room for the frit and the negative effect of releasing the pressure after packing is complete (the packing materials will pull back a little after the pressure is released).

Frits

A "frit kit" was used to make frits for fused silica capillary nano columns. A solution of 6 µL Kasil 1624 and 2 µL formamide was prepared in a safe lock tube and mixed together. The tip of a fused silica capillary was dipped into the solution for 3-5 seconds allowing the polymerization solution to migrate into the capillary by capillary forces. The fused silica capillary was then heated in a GC oven at 100 °C for at least 4 hours (preferably overnight). The end of the capillary containing the frit was then cut to 1 mm.

Washing with ACN

The column was placed in a pressure bomb system, illustrated in ***Figure 19***, where ACN was washed trough the capillary for approximately 5 minutes. When nitrogen gas is turned on it exerts a pressure on the washing solution in the vial. This forces the solution to leave the capillary towards the end containing the frit.

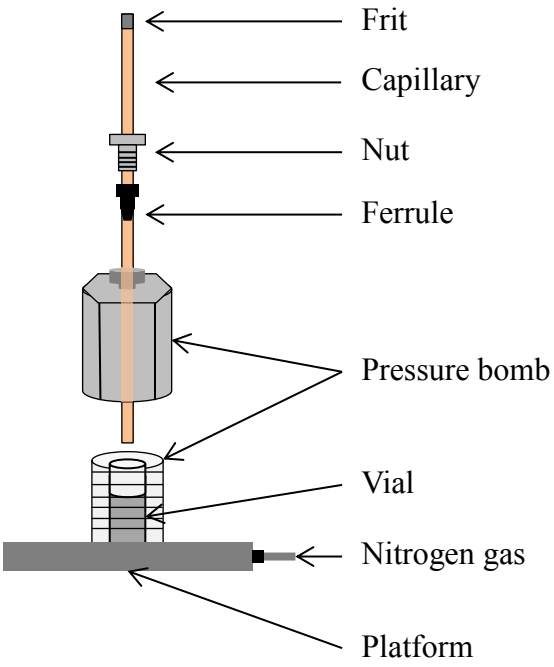


Figure 19: *An illustration of the pressure bomb system used to pack nano columns.*

Preparation of slurry

The slurry was prepared by mixing 30 mg SP in a 1.5 mL glass vial with 1 mL ACN/H_2O (80/20 *v/v*) solution. A small stirring magnet was added to the glass vial. The slurry in the glass vial was mixed and placed in an ultrasonic bath for 10 minutes.

Packing of nano columns

The packing of nano columns were done by using the pressure bomb system as illustrated in ***Figure 19*** and as described by Rogeberg *et al*. [121]. The glass vial containing the slurry (with a stirring magnet) was placed inside the pressure bomb system and the platform was placed on

a magnetic stirrer. The magnetic stirrer was on during the packing process to make sure the particles were suspended in the slurry at all time. Nitrogen gas was used to exert a pressure on the slurry and which consequently forced the slurry into the fused silica capillary towards the frit to pack the column. A microscope was used to monitor the packing process as the fused silica capillary was gradually packed.

2.5 Liquid chromatography mass spectrometer instrumentation

Two different MS instruments with different chromatographic (pumping) systems and ionization sources were used in this study and are described below. The TSQ Vantage MS was used for method development and investigation of columns with ID ≥ 0.3 mm. The TSQ Quantiva MS with nano-LC was used when investigating in-house packed nano column with 0.1 mm ID. Optimization of the MS and the ESI parameters was done with direct infusion (with a syringe and a syringe pump operated at 3 µL/min) of a standard solution containing 14 µg/mL derivatized 25-OHC.

2.5.1 Instrumentation for conventional, microbore and capillary columns

Dionex UltiMate 3000 HPLC system

A Dionex UltiMate 3000 HPLC system from Thermo Scientific was used together with the TSQ Vantage MS and includes MP reservoirs, a pump with degasser, a column oven and an autosampler as shown in ***Figure S 1*** in the appendix. The Dionex UltiMate 3000 pump is able to mix up to 4 different MP simultaneously. See the appendix ***Section 6.2*** for more information about flow rate capability and temperature range.

Agilent capillary pump and degasser

A G1376A Agilent capillary pump was used as an alternative pump (more stable at low flow rates) when investigating column with 0.3 mm ID.

TSQ Vantage mass spectrometer with heated electrospray ionization

A TSQ Vantage MS was used with a heated electrospray ionization source (HESI-II source), both from Thermo Scientific, for conventional and capillary columns with ID of 2.1 to 0.3 mm. The MS was operated in positive ionization mode with a spray voltage of 3500V and was used in selected reaction monitoring (SRM) mode as illustrated in ***Figure S 3.***

Thermo Scientifics recommendations for initial settings for the HESI-II source are shown in ***Table 8.*** These settings are based on a 50 percentage aqueous solution. When a higher amount of organic solvent in the MP was used a lower temperature and gas were applied. The position of the electrospray needle was unchanged throughout the study where the front-to-back position was 1.75 µm and the probe depth was set to position D.

Table 8: *Overview of the initial tune settings recommended from Thermo Scientific (auxiliary (aux) gas flow, sheath gas, capillary temperature etc) for different flow rates:*

Liquid flow rate	**5 µL/min**	**200 µL/min**	**500 µL/min**	**1000 µL/min**
Spray voltage (V)	3500	3500	3500	3500
Capillary temperature (°C)	240	350	380	400
Vaporizer temperature (°C)	Off to 50	250 to 350	300 to 500	500
Sheath gas (psi)	5	35	60	75
Aux gas flow (arbitrary units)	0	10	20	20

Connections and couplings for the chromatographic system and the TSQ Vantage MS

The IDs and lengths of tubings used for connecting the microbore columns (ID ≥ 0.5 mm) are shown in ***Figure 20***, including the different types of couplings. When investigating capillary columns (ID = 0.3 mm), the layout in ***Figure 21*** was used with a G1376A Agilent capillary pump.

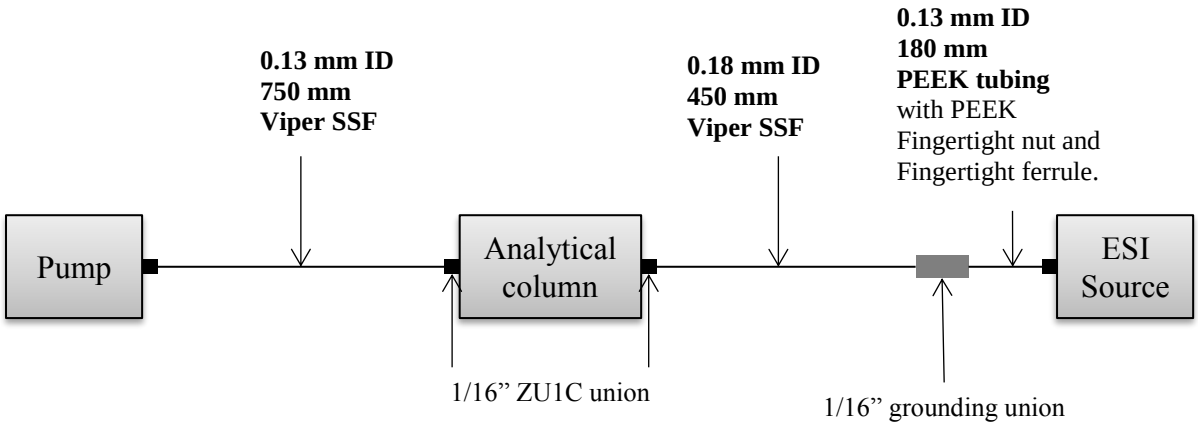


Figure 20: *The lengths and inner diameters of connections between the Dionex pump and the HESI-II source for microbore columns (ID ≥ 0.5 mm) including the different types of couplings.*

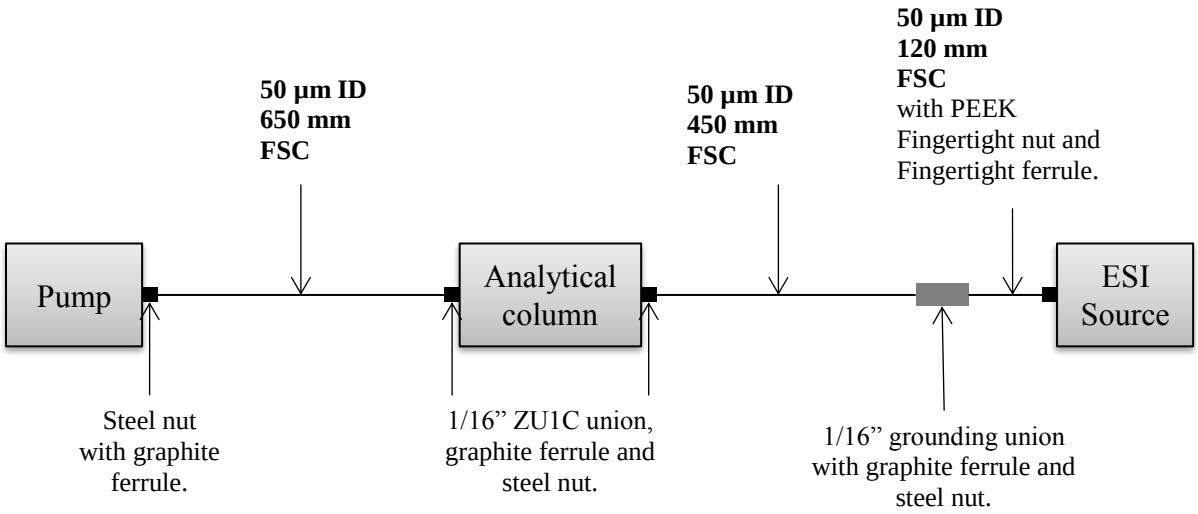


Figure 21: *The lengths and inner diameters of fused silica capillaries between the Agilent capillary pump and the HESI-II source for capillary columns (ID = 0.3 mm) including the different types of couplings.*

2.5.2 Instrumentation for nano columns

EASY-nLC 1200 system

The nanoLC 1200 system from Thermo Scientific was used together with TSQ Quantiva MS which includes MP reservoirs, pump and autosampler as shown in ***Figure 22*****.** The pump has two MP channels. This pump does not have an embedded degasser, so all MPs had to be

degassed in an ultrasonic bath for 15 minutes before use. An external column oven from Spark Mistral was used to control the column temperature.

Mobile phase reservoirs

Pump

Autosampler (built in)

Figure 22: *The nanoLC 1200 system with MP reservoirs, pump and autosampler.*

TSQ Quantiva mass spectrometer with nano electrospray ionization

The TSQ Quantiva MS with the nano flex electrospray ionization source (nLC-ESI), both from Thermo Scientific, was used when the in-house packed nanoLC columns with ID of 0.1 mm were investigated.

The MS was operated in positive ionization mode with a spray voltage between 1800-1900V and a capillary temperature between 310 – 340 °C. The electrospray needle was positioned 2-5 mm from the MS inlet. The ionization source did not have any sheath or aux gas options in the configuration used in this thesis. The MS was operated in SRM mode as illustrated in ***Figure S 3.***

Connections and couplings for the chromatographic system and the TSQ Quantiva MS

The inner diameters and lengths of the nanoViper and the fused silica capillary for the connection of the nanoLC 1200 pump to the TSQ Quantiva for investigation of nano columns (ID = 0.1 mm) are shown in ***Figure 23***, including the different types of couplings.

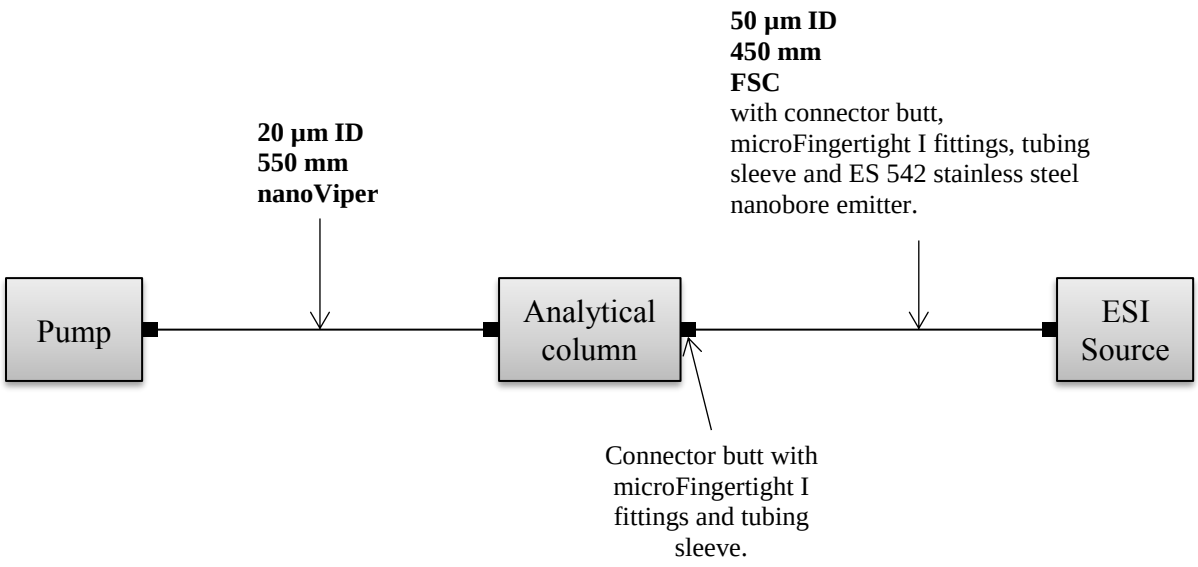


Figure 23: *The lengths and inner diameters of connections between the nanoLC 1200 pump and the nLC-ESI for columns with ID = 0.1 mm including the different types of couplings.*

The connections used when the manual injector was implemented together with the chromatographic system for investigating of nano column, the layout in ***Figure 24*** was used and includes the different types of couplings.

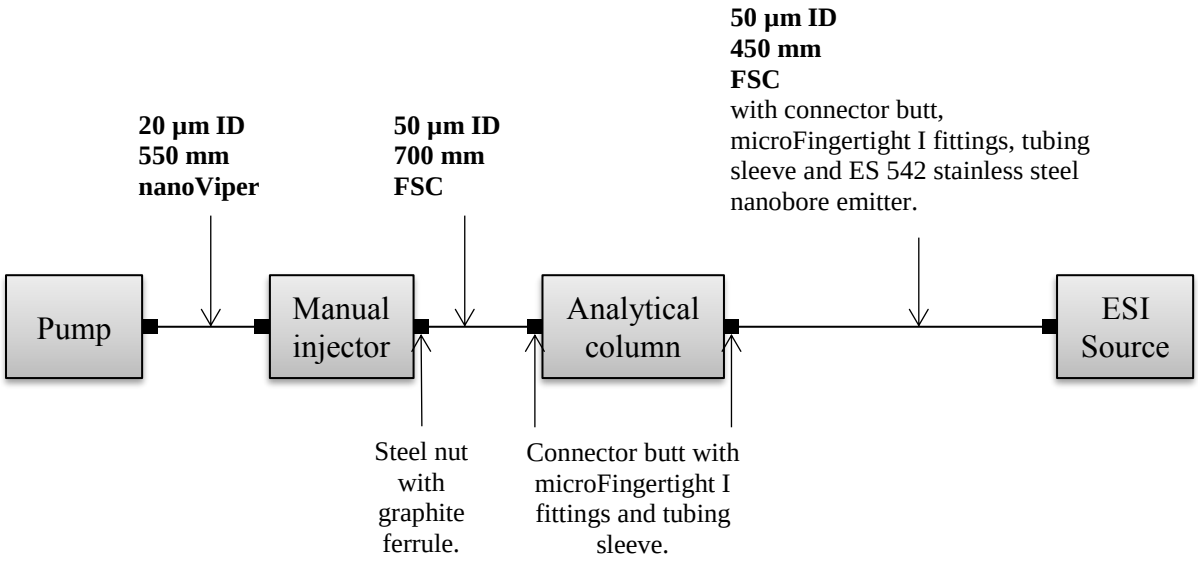


Figure 24: *The lengths and inner diameters of connections between the nanoLC 1200 pump and the nLC-ESI for columns with ID = 0.1 mm when the manual injector was implemented and includes the different types of couplings.*

An illustration of the Cheminert manual injector from Vici Valco with 50 nL injection loop is shown in ***Figure 25***.

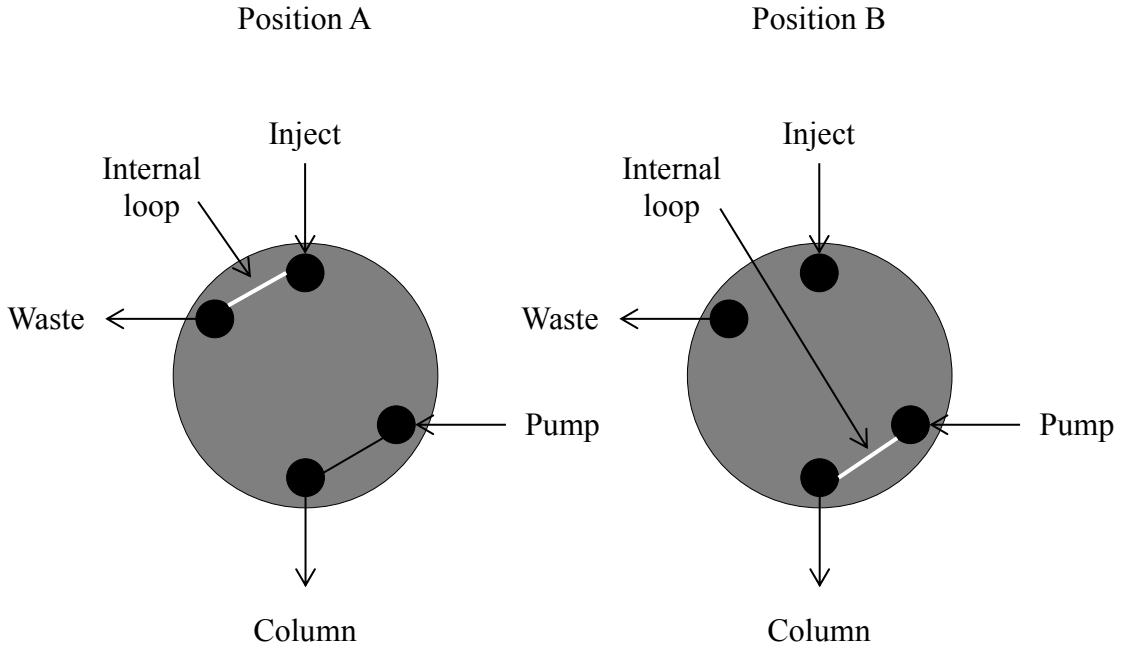


Figure 25: *An illustration of how the 2-way-4-port manual injector with internal loop works. Position A (loading position) is used to load the internal loop with the sample. Position B (inject position) transports the sample from the internal loop to the inlet of the column.*

2.5.3 Software

The Thermo Xcalibur software (Xcalibur) (program version 2.0) was used for the method- and sequence setup for both the TSQ Vantage and the TSQ Quantiva MS. Xcalibur was also used for data handling. The Dionex Ultimate 3000 pump with column oven and autosampler was controlled with Chromeleon Express (program version 6.80 SR13 Build 3818). Thermo TSQ Tune Master (program version 2.3.0.1214 SP3) was used to control the TSQ Vantage MS equipped with the HESI-II source. The TSQ Quantiva tune application (program version 1.1.1031) was used to control the TSQ Quantiva equipped with the nLC-ESI source. The nanoLC 1200 pump with autosampler was controlled directly by the touch screen.

3 Results and discussion

In the present study, development and optimization of a new method for determination the side-chained oxysterols 22R-, 24S-, 25-, 27- and 22S-OHC have been the main focus. The aim has been to decrease analysis time so that the method could be used *e.g.* towards diagnostic screening of oxysterols where the limiting factor today is the analysis time. With this intention in mind, different commercially available columns (both with totally porous and core-shell particles) have been thoroughly investigated and optimized considering the different chromatographic conditions (flow rate, temperature and MP composition). The chromatographic conditions was also fine-tuned for the columns that showed potential regarding separation of the oxysterols within a reasonable time. In addition, the column with the stationary phase that demonstrated the greatest chromatographic performance (*i.e.* highest resolution within acceptable analysis time) was emptied and the SP from the column was re-packed into another column body with a more narrow ID to enhance the sensitivity.

All development and optimization have been carried out with commercially obtained standards which have underwent the sample preparation (oxidation and derivatization) as described in ***Section 6.1.1*** and ***6.1.2*** before chromatographing and detection with MS. See the appendix ***Section 6.3*** for interpretation of the chromatograms.

3.1 Optimization of the mass spectrometer and the electrospray ionization source with direct infusion

The different settings for the MS-ESI (*e.g.* electrospray voltage, fragmentation energy, position for the MS lenses *etc*.) need to be optimized before determination of oxysterols. This was done by direct infusion (with a syringe coupled directly to the ESI source) of a solution containing one of the analyte investigated. The preparation of the standard solution containing the analyte was the same as described in ***Section 6.1.1*** and ***6.1.2.*** The results from MS-ESI optimization are given in the appendix in ***Section 6.4*** and an overview of the *m/z* transitions that the MS was targeted towards is given in ***Table 9***. Girard T derivatives provide distinct fragments (***Figure S 2*** in the appendix) of -59 Dalton (Da) and -87 Da and a suggested fragmentation reaction is shown in ***Figure 26*** [44, 55, 116]. The *m/z* transitions that were observed for the oxysterols were the same *m/z* transitions that have been reported in previous work.

Table 9: *The monitored m/z transitions for Girard T derivatives of hydroxycholesterols in MS and MS/MS mode.*

Analyte	*m/z* MS mode	Fragmentation	*m/z* MS/MS mode
22R-OHC	514.4	514.4 → 455.4	455.4 and 427.4
24S-OHC	514.4	514.4 → 455.4	455.4 and 427.4
25-OHC	514.4	514.4 → 455.4	455.4 and 427.4
27-OHC	514.4	514.4 → 455.4	455.4 and 427.4
22S-OHC	514.4	514.4 → 455.4	455.4 and 427.4

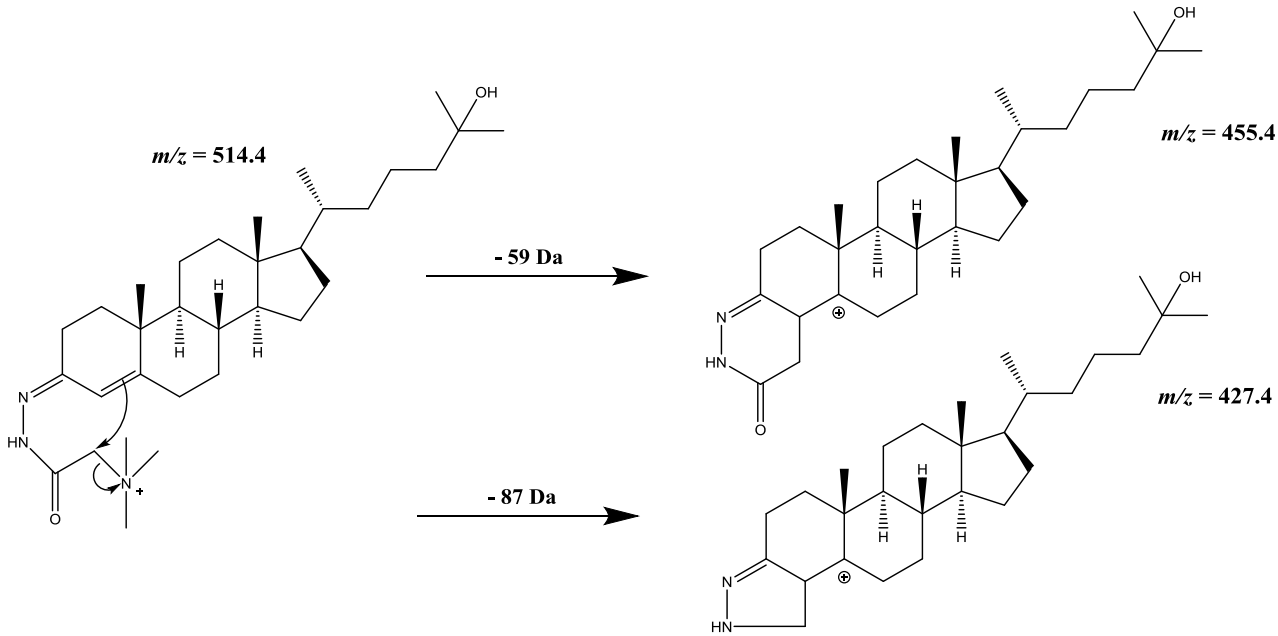


Figure 26: *A suggested fragmentation reaction of Girard T derivatives of oxysterols in MS/MS, here illustrated with 25-OHC.*

3.2 Investigation of column performance

Several columns were examined during the method development. The Hypersil Gold C18 column and the two columns from ACE went through a systematic sequence where different conditions were explored. The conditions were then fine-tuned for each column depending on how they performed chromatographically. The conditions that were taken into account was the type of organic solvent, the ratio between the organic solvent and the water, flow rates and the temperature in the column oven.

3.2.1 Columns containing totally porous particles

Investigation of the Waters columns

Jumaah *et al.* and Sandvik *et al.* have used the 2-PIC and 1-AA columns from Waters for the separation of vitamin D [122, 123] which has a similar structure as the oxysterols. However, the 2-PIC and 1-AA Waters columns were not suited for the separation of the isomeric oxysterols in RPLC mode and the results from these two columns are found in the appendix in ***Section 6.7.1***.

Investigation of a Thermo Hypersil Gold C18 column

The Thermo Scientific Hypersil Gold C18 column has totally porous particles with a C18 SP that provides hydrophobic interactions towards hydrophobic compounds. The main difference between the oxysterols is where the hydroxyl group is attached on the side-chain (***Figure 2***). Previous methods for separation of the oxysterols by our group have been performed with analytical columns with a C18 SP. Examination of different SPs has been one of the main approaches for the separation of oxysterols in our group [83, 124]. However, the optimal chromatographic performance have usually achieved with a C18 SP, where the Thermo Hypersil Gold column has been a favorite for several oxysterol analysts [44, 52, 55, 103-105].

The Thermo Hypersil Gold column provided separation between the enzymatically formed oxysterols (not 22S-OHC) within an analysis time of ≈10 minutes, and which was an improvement compared to previous methods [52, 116]. The improved analysis time was achieved by decreasing the column temperature to 15 °C. The optimal column performance achieved when investigating the Hypersil Gold column is shown in ***Figure 27***. However, the analysis time was not acceptable (>5 minutes) with the optimal conditions that were found. Hence, it was decided to investigate columns from ACE with core-shell particle.

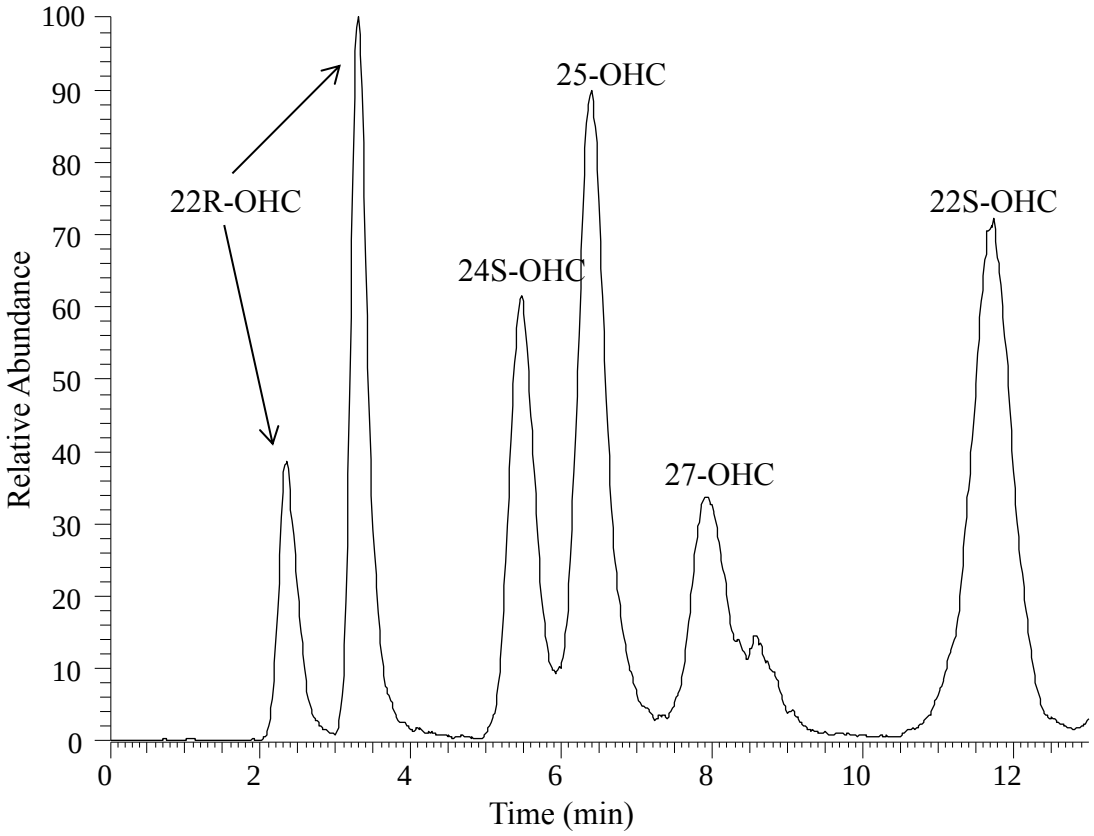


Figure 27: *A TIC chromatogram (7 points smoothing) of m/z 514.4 → 455.4 of a standard solution containing 22R-, 24S-, 25-, 27- and 22S-OHC (2.8 µg/mL each). The separation was done with a 1.0 mm ID Thermo Hypersil Gold C18 column by using the system described in* ***Section 2.5.1****. The flow rate was 110 µL/min at 15 °C where the MP consisted of H_2O/MeOH 30/70 (v/v) with 0.1% FA.*

More results for the Thermo Scientific Hypersil Gold C18 column are presented in the appendix in ***Section 6.7.2***.

3.2.2 Columns containing core-shell particles

The ACE UltraCore SuperPhenylHexyl column (SPH) and the ACE UltraCore Super C18 column both have core-shell particles (***Section 1.4.2***). This enable the possibility to utilize a higher flow rate without overloading the pump with regard to the increased backpressure, which is a common problem with totally porous particles with the same particle size and chromatographic set-up [89, 91, 125]. Both of the ACE columns have 2.5 µm core-shell particles which provide approximately the same efficiency as sub-2 µm totally porous particles

[89]. In addition, core-shell particles with an diameter of 2.5 – 2.7 µm have approximately one-half to one-third of the operating pressure compared with sub-2 µm totally porous particles [126]. The diffusion of compounds in a thin porous layer gives higher separation efficiency (with same particle diameter and chromatographic conditions) and less band broadening compared to a totally porous particle [58, 127]. The columns from ACE with totally porous particles and various dimensions have been used several times for the separation of oxysterols in our group [56, 57, 82, 128]. In our hands, ACE provides quality columns and is a brand that we are familiar with, and was the reason why columns from ACE with core-shell particles were selected for investigation in this thesis (and not *e.g.* Kinetex™ or Halo® columns).

Investigation of the ACE UltraCore Super C18 column

One of the reasons why this column was selected as a candidate for the separation of the oxysterols was that the SP is similar to that has been used before (*e.g.* The Hypersil Gold column) where a C_{18} SP successfully separated the analytes.

A systematical investigation of the ACE UltraCore SuperC18 column was performed. The amount of MeOH in the MP, the flow rate and the temperature were examined without success. This systematical investigation is shown in ***Table S 1*** in the appendix. A step-by-step investigation of the ACE UltraCore SuperC18 column was performed with several different conditions where some examples are given in ***Table S 2***.

The peaks for the oxysterols 22R-, 27-, and 22S-OHC appears as two separate peaks in the chromatograms due to stereoisomerism [124] which is one of the drawbacks of using Girard reagents for derivatization. The derivatization reaction is not stereospecific, hence c*is* (or Z) and *trans* (or E) isomers are introduced. This might give rise to two chromatographically separated compounds for some of the oxysterols using LC-MS depending on the chromatographic conditions used [52, 129].

The oxysterols 24S- 25- and 27-OHC co-eluted for almost every conditions that were examined (the amount of MeOH, different flow rates and temperatures). Different gradient elution were examined where the amount of MeOH in the MP was changed over time without success. Both elevated temperatures (70 – 80 °C) and temperatures below room temperature (10 - 15°C) did not improve the separation (chromatograms not shown). Also, different amounts of ACN as the

organic solvent (instead of MeOH) in the MP was examined without success. Several MP compositions that consisted of a mixture of ACN and MeOH as the organic solvent were examined (see the appendix ***Table S 3)***. The effects of adding ACN to the mixture together with MeOH as organic solvent shortens the retention times considerably. For 27-OHC the difference in the retention time was 4.0 minutes when increasing the amount of ACN in the MP mixture with 2% at the same flow rate and temperature. However, none of the MP compositions consisting of MeOH and ACN provided the desired resolution ($R_S \approx 1.5$) nor the analysis time (< 5 minutes).

The best resolution between 24S-, 25-, and 27-OHC using the ACE Super C18 column was achieved with H_2O/MeOH/ACN 31/67/2 (*v/v/v*) with 0.1% FA as the MP composition at a flow rate of 440 µL/min at 30 °C. A representative chromatogram is shown in ***Figure 28***. The resolution between 24S-OHC and 25-OHC and 25-OHC and 27-OHC was 0.8 and 1.2, respectively.

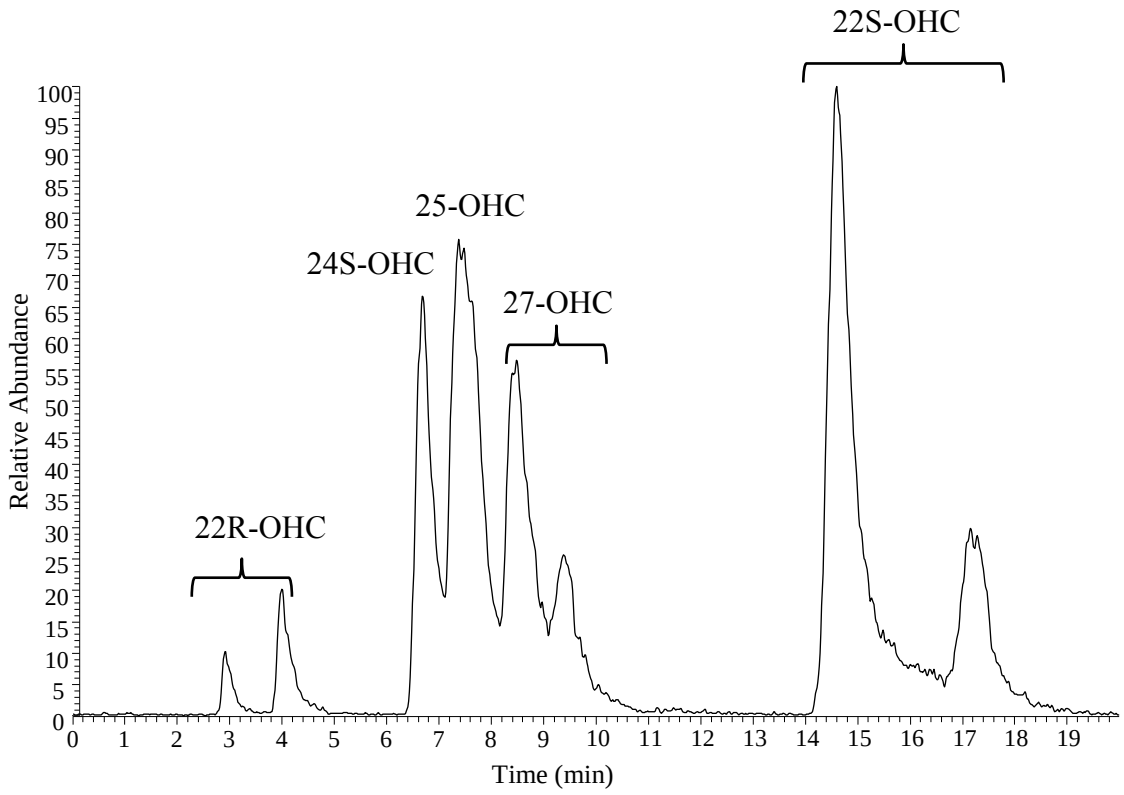


Figure 28: *A TIC chromatogram (7 points smoothing) of m/z 514.4 → 455.4 of a standard solution containing 22R-, 24S-, 25-, 27- and 22S-OHC (2.8 µg/mL each). The separation was done with the ACE Super C18 column by using the system described in* ***Section 2.5.1****. The MP composition was H_2O/MeOH/ACN 31/67/2 (v/v/v) with 0.1% FA with a flow rate of 440 µL/min at 30 °C.*

To sum up, the ACE Super C18 column did not provide the same resolution between the oxysterols as the Hypersil Gold column. The resolution and analysis time were not acceptable with the conditions used to obtain the chromatogram in ***Figure 28.*** *Hence, after extensive examination of different conditions (different flow rates, temperatures, different organic solvents and different MP compositions) it was concluded to not move forward with the ACE Super C18 column.*

Investigation of the ACE UltraCore SuperPhenylHexyl column

The SP of the ACE SPH column consist of an alkane chain and a phenyl group as illustrated in ***Figure 13***, and can provide an alternative interaction (*e.g.* π-π-interactions) compared to columns with C18 SP. A systematical investigation of the ACE UltraCore SPH column was performed, where the amount of MeOH in the MP, flow rates and temperatures were examined without success. This systematical investigation is shown in ***Table S 4*** in the appendix. General effects of the amount of MeOH in the MP, the effect of adjusting the temperature and the effect of different flow rates are presented in ***Section 6.7.3*** in the appendix.

Previous work has shown rapid determination of sterols (with various resolution though) where columns with phenyl hexyl SP have been used [39, 106, 130-133]. The rapid determination of side-chained oxysterols have been achieved by using a mixture of ACN and MeOH as organic solvent in the MP. Mixtures of ACN and MeOH were not taken into consideration in the systematical investigation. Further examination of the ACE SPH column was therefore performed to investigate the effect of different mixtures of ACN and MeOH as the organic solvent in the MP.

Effect of mixing MeOH and ACN as MP

Pataj *et al*. [106] separated 24S-, 25-, and 27-OHC within 4 minutes using a Kinetex™ 2.6 μm Biphenyl column (also with core-shell particles). However, the derivatization method used by Pataj *et al*. for determination of side-chained oxysterols was N,N-dimethylglycine (DMG) which resulted in high detection limits (1–4.5 ng/mL) compared to previous work in our group using Girard T as derivatization reagent (0.005–0.01 ng/mL) [57].

The Kinetex™ 2.6 µm Biphenyl SP consists of two phenyl groups, while the SP for the ACE SPH column have one phenyl group, as illustrated in ***Figure 29***. This means that it could be possible to separate the oxysterols by other interactions (*e.g.* π-π interactions towards the double bond in the sterol ring) that occur between the SP and the oxysterols, and not only, as previously thought when investigating the Hypersil GOLD C_{18} column, by the hydrophobic interactions towards the side chain of the oxysterols.

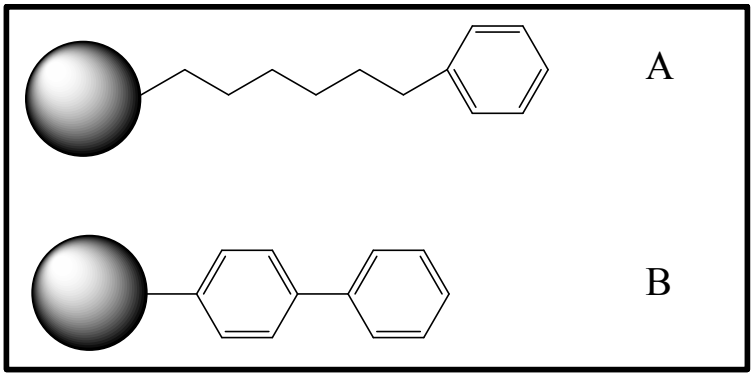


Figure 29: *An illustration of the SP for* ***A)*** *ACE SuperPhenylHexyl and* ***B)*** *Kinetex™ Biphenyl.*

Pataj *et al*. used gradient elution at 30 °C with the MP A consisting of MeOH/H_2O (5/95 (*v/v*)) and the MP B consisting of MeOH/ACN (10/90 (*v/v*)), both with 0.1% FA and 2 mM ammonium acetate. The gradient elution started with 42% of MP B with a flow rate of 500 µL/min with a linear increase to 50% MP B in 3.0 minutes (the gradient in the paper also included a washout step).

In our hands, using the ACE SPH column and the same chromatographic conditions (without ammonium acetate since FA secures the charge needed for MS-detection), some separation was achieved, but not base line separation ($R_S \ll 1.5$) as seen in ***Figure 30***. This indicated that the analytes have less affinity towards the ACE SPH SP relative to the Kinetex Biphenyl SP or because of the different derivatization steps used (here Girard T instead of DMG), either way, the amount of organic solvent in the MP had to be reduced.

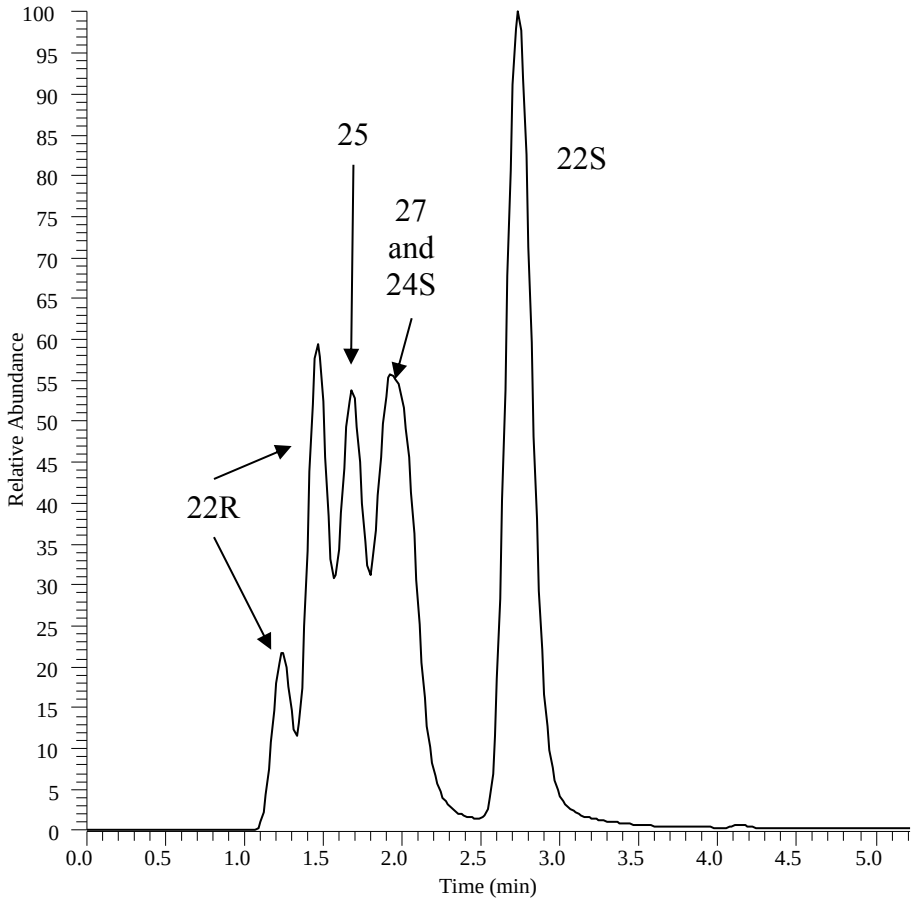


Figure 30: *A TIC chromatogram (7 points smoothing) of m/z 514.4 → 455.4 of a standard solution containing 22R-, 24S-, 25-, 27- and 22S-OHC (2.8 µg/mL each). The separation was done with the 2.1 mm ID ACE SPH column by using the system described in* ***Section 2.5.1****. The MP A consisted of MeOH/H_2O 5/95 (v/v) and the MP B consisted of MeOH/ACN 10/90 (v/v), both (A and B) with 0.1% FA. The gradient elution started at 42% B with a flow rate of 500 µL/min at 30 °C with a linear increase to 50% B in 3.0 minutes.*

Different gradients elutions, isocratic elution conditions, flow rates and temperatures were examined (see ***Table S 5***, ***Table S 6*** and ***Table S 7*** in the appendix). The best resolution ($R_S \approx 1.5$) was achieved when using an isocratic elution with 35% B with a flow rate of 800 µL/min at 50 °C. The MP A consisted of MeOH/H_2O 5/95 (v/v) and the MP B was MeOH/ACN 10/90 (v/v), both (A and B) with 0.1% FA.

The H_2O, MeOH and ACN were filled into separate solvent reservoirs to achieve a 3-solvent MP (instead of pre-mixing them). Hence, each solvent came from different pumping channels; channel A was H_2O, channel B was MeOH and channel C was ACN, all with 0.1% FA. This set-up allowed the possibility to fine tune the amount of H_2O, MeOH and ACN (see ***Table S 8***

and ***Table S 9*** in the appendix for some of the experiments conducted). Experiments with fine tuning of the MP composition revealed that the *best chromatographic performance was achieved by a flow rate of 900 µL/min and a temperature of 45 °C with a MP composition consisting of H_2O/MeOH/ACN 60/8.5/31.5 (v/v/v) (with 0.1% FA).* A representative chromatogram with these condition is shown in ***Figure 31***. The efficiency is calculated for 22R-, 25-, 24S- and 27-OHC in ***Table 10.***

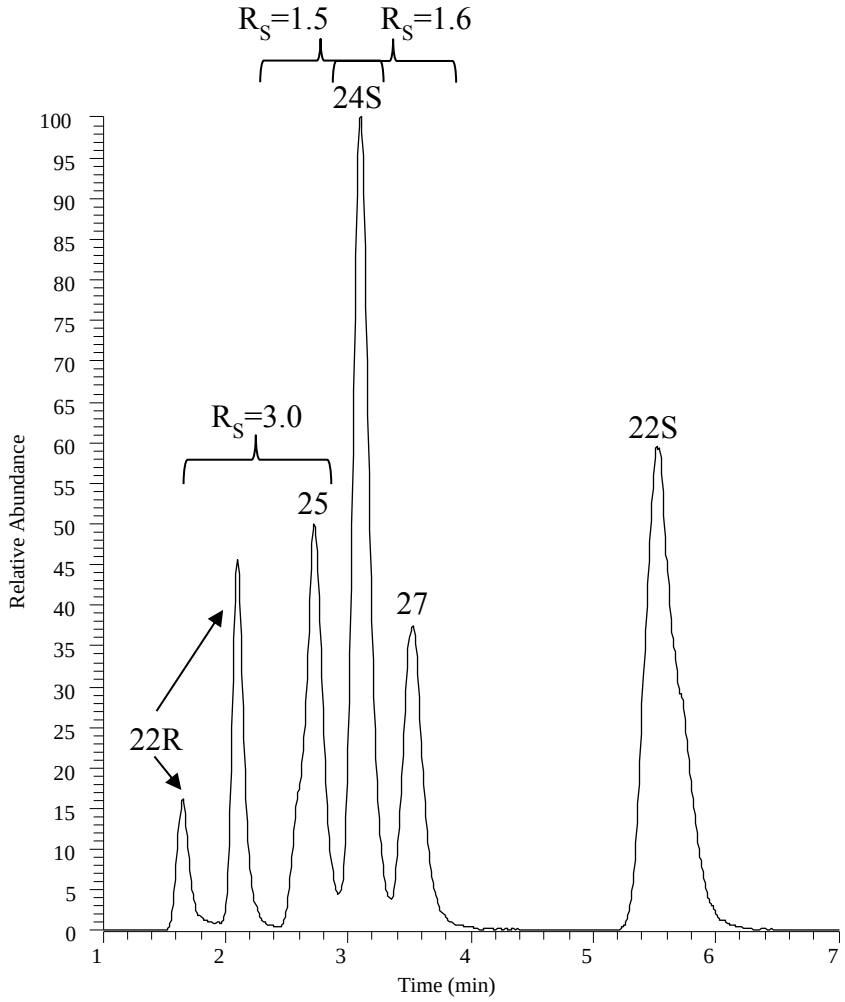


Figure 31: *A TIC chromatogram (7 points smoothing) of m/z 514.4 → 455.4 of a standard solution containing 22R-, 24S-, 25-, 27- and 22S-OHC (2.8 µg/mL each). The separation was done with the 2.1 mm ID ACE* SPH *column by using the system described in* ***Section 2.5.1****. The MP composition consisted of H_2O/MeOH/ACN 60/8.5/31.5 (v/v) with 0.1% FA. The flow rate was 900 µL/min at 45 °C.*

Table 10: *The efficiency for each peak (analyte) with the conditions used to obtain the chromatogram in* ***Figure 31****.*

Analyte	**Efficiency (calculated using *Equation 2* and the chromatogram in *Figure 31)***
22R-OHC	N = 2440
25-OHC	N = 1700
24S-OHC	N = 2530
27-OHC	N = 2750

Note that the retention order between 24S-OHC and 25-OHC shifted when ACN and MeOH was mixed together as the organic solvent. By comparing the results obtained by *e.g.* Pataj *et al.* [106] compared with the results obtained by Honda *et al.* [52] for determination of the same oxysterols, the same shift between 24S-OHC and 25-OHC is observed.

The experiments conducted with the 2.1 mm ID ACE SPH column also showed that elevated temperatures did not affect the resolution to the same extent as it did for the columns with C18 SP when the MP mixtures of MeOH and ACN were used. An example for the ACE SPH column is given in ***Figure S 5*** in the appendix where the conditions used to obtain the chromatogram **E** in ***Figure S 5*** provides a faster analysis time compared to that achieved by Pataj *et al*. [106]. However, since the resolution between 25- and 24S-OHC and 24S- and 27-OHC in chromatogram **E** were 1.4 and 1.3 at this temperature, the conditions used to obtain ***Figure 31*** were still the best option to go forward with.

To sum up; by using a mixture of MeOH and ACN as the MP and a high flow rate at 45 °C, the ACE SPH column provided the analysis time and resolution needed for the determination of oxysterols in a diagnostic screening setting. Based on these results, the ACE SPH column was selected for further study. In order to achieve a higher sensitivity, a more narrow column should be used. However, since such column was not commercially available, a second option was to re-pack the SP into more narrow format.

3.3 Downscaling the chromatographic system

Oxysterols are present in plasma in the low ng/mL range and there are limited sample amounts. By minimizing the column ID, a higher theoretical sensitivity can be obtained ***(Figure 11***) when using a concentration sensitive detector, such as ESI coupled to MS and which enables the possibility to detect the low concentration of oxysterols in plasma. Hence, in-house packed column were made with the SP from the ACE SPH column with serial no: A174508.

Three different ID of in-house packed column were explored it this study, 0.5 mm, 0.3 mm and 0.1 mm ID, all of them packed with the SPH SP. The columns with ID of 0.5 mm and 0.3 mm were packed in stainless steel column body, while the 0.1 mm ID column was packed in a fused silica capillary (FSC). The stainless steel column body is easier to handle in regards to couplings and detection of leakages and experience indicate it can withstand a higher pressure. This is because the connections and couplings used in this study for the stainless steel columns are more robust than the PEEK sleeves and the PEEK connections used for the nano-columns (FSC). Nano-vipers were not available at the time the nano-columns were investigated. Moreover, the packing procedure for stainless steel columns is less time consuming relative to the packing procedure for nano-columns, and it does not require constant monitoring during the process.

The results for each in-house packed column are presented in the chapters below. Later, an ACE SPH column with 0.5 mm ID became commercially available and the performance of this column is presented in ***Section 3.3.4.*** In ***Table 11*** below is an overview of the in-house packed columns with the theoretically increased sensitivity together with a calculated flow rate (***Equation 10***) to obtain the same linear velocity found for the 2.1 mm ID ACE SPH column that gave the best chromatographic performance.

Table 11: *An overview of the ID for the in-house packed columns with the theoretical increase sensitivity and calculated flow rate in regards to the best chromatographic performance found for the 2.1 mm ID ACE SPH column.*

Inner diameter	Increased sensitivity (theoretical) relative to the 2.1 mm ID column	Optimal flow rate
0.5 mm ID	17.6	51 µL/min
0.3 mm ID	49	18.4 µL/min
0.1 mm ID	441	2.0 µL/min

3.3.1 In-house packed 0.5 mm ID column

Three attempts were made for packing 0.5 mm ID columns (procedure described in ***Section 2.4.1***), where the first two were unsuccessful due to technical problems. One of the major challenges in the packing of column is to achieve a uniform and densely packed bed with few defects and voids. The packing of columns has been studied in detail where different parameters (*e.g.* temperature, packing pressure, slurry concentration (particles/mL solvent), solvent used, *etc.*) have been discussed with regards to the effect these parameters have on the performance on a packed column [76, 134, 135]. To pack a well packed column requires experience to succeed.

The third attempt to pack a 0.5 mm ID column went well considering the packing procedure. The column showed better stability than the two first columns, but provided poor chromatography with wide peaks where several analytes co-eluted. Several experiments were conducted to improve the resolution, but without success. A representative chromatogram for each in-house packed column is shown in ***Figure 32***.

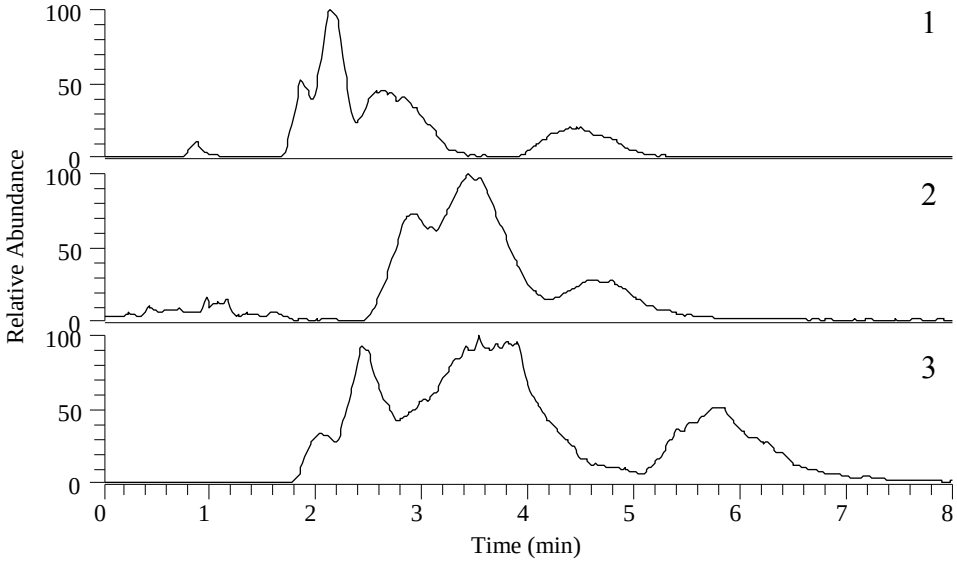


Figure 32: *TIC chromatograms (7 points smoothing) of m/z 514.4 → 455.4 of a standard solution containing 22R-, 24S-, 25-, 27- and 22S-OHC (2.8 µg/mL each). The separation was done with three different in-house packed 0.5 mm ID columns with the SPH SP by using the system described in Section 2.5.1. The MP composition consisted of H_2O/MeOH/ACN 60/8.5/31.5 (v/v/v) with 0.1% FA. The flow rate was 50 µL/min at 45 °C. The numbers in the chromatograms illustrate the column (first, second, third) that were used to obtain the chromatogram.*

Thus, no further investigation was done of the in-house packed 0.5 mm ID columns based on their poor chromatographic performance.

3.3.2 In-house packed 0.3 mm ID column

The column was packed with the ACE SPH particles with the packing procedure as described in ***Section 2.4.1***.

The optimal flow rate for the 0.3 mm ID column should be approximately 20 µL/min according to ***Equation 10*** and ***Table 11***. At this flow rate and with a MP consisting of H_2O/MeOH/ACN 60/8.5/31.5 (*v/v/v*) at 45 °C the resolution was poor ($\ll 1.5$) where 24S-, 25- and 27-OHC co-eluted (chromatogram not shown).

The amount of organic solvent in the MP was decreased as an attempt to separate the co-eluting oxysterols 24S-, 25- and 27-OHC. With a MP consisting of H_2O/MeOH/ACN 61/8/31 (*v/v/v*)

at 50 °C and a flow rate of 25 µL/min, an improved resolution between the co-eluting oxysterols was achieved, but with an $R_S < 1.0$ and at the cost of increased analysis time (shown in ***Figure S 6*** in the appendix).

The flow rate for the in-house packed 0.3 mm ID column was examined from 10 to 30 µL/min, and as described in the appendix ***Section 6.2***, the Dionex UltiMate 3000 HPLC pump can give unstable supply of MP when it is operated below 50 µL/min flow. The Agilent capillary pump is able to deliver a more stable flow rate in the examined flow rate region. The drawback with this pump is the maximum of two pumping channels. With only two pumping channels, a mixture of H_2O/MeOH/ACN had to be made manually where pump A delivered a pre-mixed composition of MeOH and H_2O (5/95 *v/v*) with 0.1% FA and pump B delivered a pre-mixed composition of MeOH and ACN (10/90 *v/v*) with 0.1% FA.

With a MP consisting of 35% B at a flow rate of 10 µL/min (upper pressure limit for Agilent pump was reached at this flow rate) and 50 °C, the resolution was improved some ($R_S < 1.0$), but not as good as the resolution obtained with the 2.1 mm ID ACE SPH column ($R_S \approx 1.5$) as shown in ***Figure 33.***

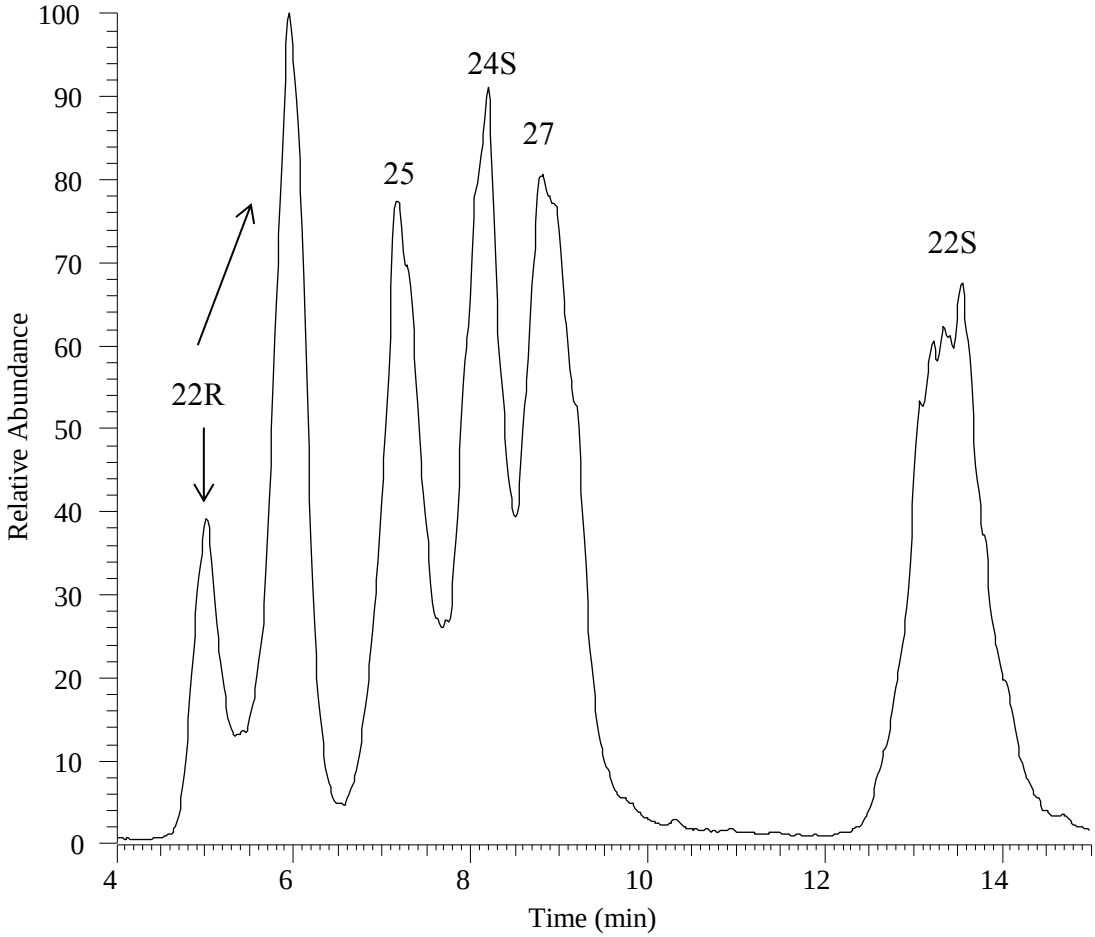


Figure 33: *A TIC chromatogram (7 points smoothing) of m/z 514.4 → 455.4 of a standard solution containing 22R-, 24S-, 25-, 27- and 22S-OHC (2.8 µg/mL each). The separation was done with an in-house packed 0.3 mm ID column with the* SPH *SP by using the system described in* ***Section 2.5.1****. The MP A consisted of MeOH/H_2O 5/95 (v/v) and the MP B consisted of MeOH/ACN 10/90 (v/v) both with 0.1% FA. The chromatogram was obtained with 35% B with 10 µL/min at 50 °C.*

Due to the pressure limitation on the Agilent pump the analysis time was three fold increased whereas the column efficiency was reduced with approximately 30% compared to the commercial 2.1 mm ID ACE SPH column, hence the 0.3 mm ID column dimension was not examined further.

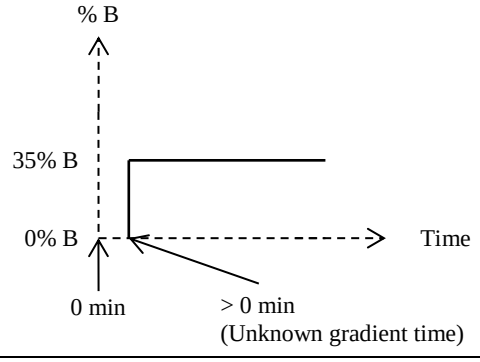
3.3.3 In-house packed 0.1 mm ID column

To maintain the same linear velocity for the in-house packed 0.1 mm ID column compared with the commercial obtained 2.1 mm ID ACE SPH column the flow rate should be 2 µL/min (***Table 11***), resulting in ≈700 bars pressure. The high pressure caused leakages between couplings in the LC system and made it impossible to uphold the same linear velocity found for the commercially obtained column. The limitation was therefore not the pump itself (the pump can withstand a pressure of 1200 bar), but the couplings in the chromatographic system.

Another challenge when using the EASY-nLC 1200 nano-LC chromatographic instrumentation is that it is designed for *gradient* elution of the MP. When the EASY-nLC pump receives settings from the Excalibur software, the pump delivers a pre-set flow from pump A as default. Pump A consist of H_2O/MeOH 95/5 (*v/v*) with 0.1% FA, and which means that the analytical column is not pre-conditioned with the desired MP composition before the analysis starts. Hence, an "unknown and not repeatable gradient elution" occurs instead of an isocratic elution.

The EASY-nLC pump which is built to deliver *gradient elution*, was used with the elution steps as shown in ***Table 12***. All chromatograms obtained with the in-house packed 0.1 mm ID column in this thesis will be represented with an "*unknown gradient elution"*.

Table 12: *The elution steps for the EASY-nLC 1200 system exemplified with an instrument method with a MP composition consisting of 35% B and 65% A. The gradient time is unknown (> 0.0 min).*

Time (min)	Amount of A (%)	Amount of B (%)	Illustration of the increase of % B in terms of time
0.0	100	0	
> 0.0 (Unknown gradient time)	65	35	

Both a systematically and a step-by-step approach were performed to investigate the in-house packed 0.1 mm ID column (see ***Table S 10***, ***Table S 11***, ***Table S 12***, ***Table S 13*** and ***Table S 14*** in the appendix).

The optimal chromatographic performance for the 0.1 mm ID column with the conditions investigated (shown in the tables mentioned above) were obtained with a MP B consisting of MeOH/ACN 20/80 (*v/v*) with 0.1% FA. A representative chromatogram is shown in ***Figure 34***. The column efficiencies calculated for 22R-, 25-, 24S- and 27-OHC are given in ***Table 13***. ***Table 10*** and ***Table 13*** shows that the efficiency for 25- and 24S-OHC was improved for the in-house packed 0.1 mm column compared to the commercially obtained 2.1 mm ID column (the column lengths for both the 0.1 mm ID column and the commercial 2.1 mm ID column were 15 cm).

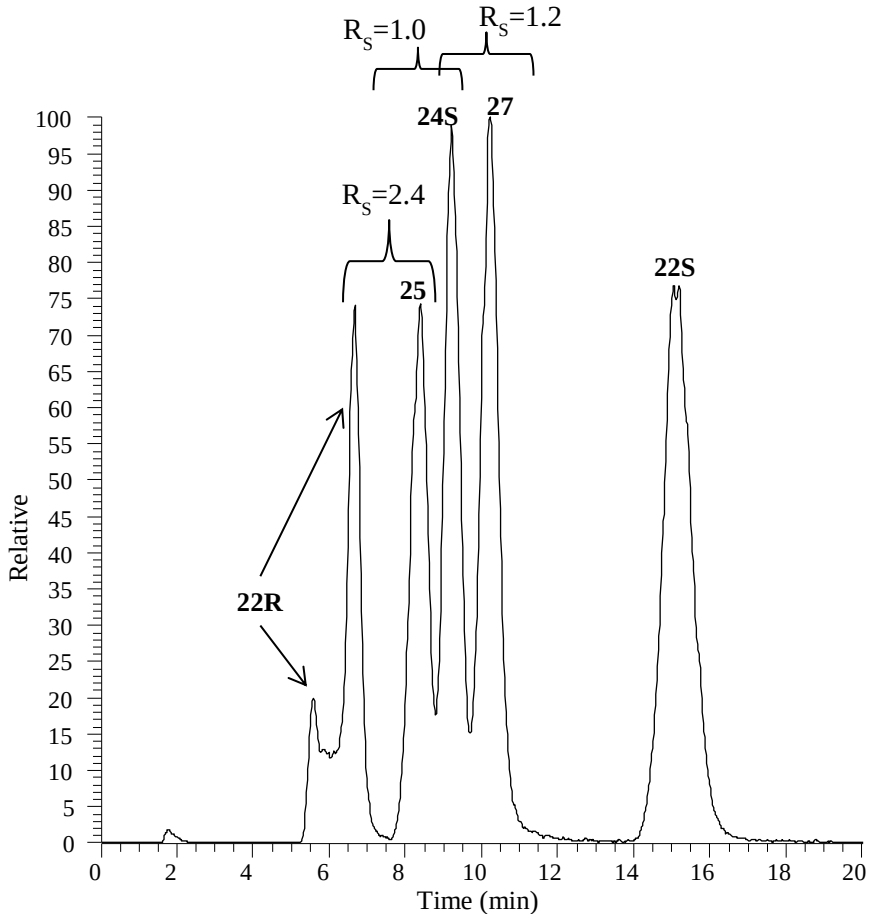


Figure 34: *A TIC chromatogram (7 points smoothing) of m/z 514.4 → 455.4 of a standard solution containing 22R-, 24S-, 25-, 27- and 22S-OHC (2.8 µg/mL each). The separation was done with an in-house packed 0.1 mm ID column with the* SPH *SP by using the system described in* ***Section 2.5.2.*** *The MP A consisted of MeOH/H_2O 5/95 (v/v) and the MP B consisted of MeOH/ACN 20/80 (v/v), both with 0.1% FA. The flow rate was 1000 nL/min with an "unknown gradient elution" ending at 28% B at 55 °C.*

Table 13: *The efficiency for each peak (analyte) with the conditions used to obtain the chromatogram in* ***Figure 34****.*

Analyte	**Efficiency (calculated using *Equation 2* and the chromatogram in *Figure 34)***
22R-OHC	N = 2415
25-OHC	N = 1760
24S-OHC	N = 2550
27-OHC	N = 2140

Thus, even though the efficiency for the 0.1 mm ID column was almost identical with the 2.1 mm ID column, the resolution and analysis time were not acceptable (R_S < 1.5 and analysis time > 5 minutes). Experiments with manual injector were conducted (set up as shown in ***Figure 24*** *in* ***Section 2.5.2****), but without improvement of the resolution, hence this column dimension was not examined further.*

3.3.4 Commercially obtained 0.5 mm ID x 150 ACE SuperPhenylHexyl

During the study, a 0.5 mm ID x 150 ACE SPH column with 2.5 µm particles became available. The column was examined with the same conditions (flow rate (linear velocity), temperature and MP composition) that gave the best chromatographic performance for the commercially obtained 2.1 mm ID column with the same SP (***Figure 31***).

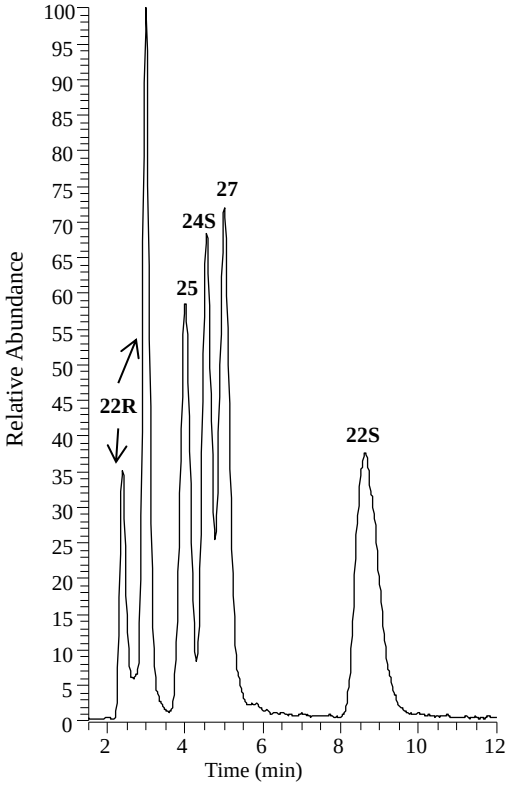


Figure 35: *TIC chromatogram (7 points smoothing) of m/z 514.4 → 455.4 of a standard solution containing 22R-, 24S-, 25-, 27- and 22S-OHC (2.8 µg/mL each). Separation was done with a commercially obtained 0.5 mm ID ACE* SPH *column and by using the system described in* ***Section 2.5.1****. MP composition was H_2O/MeOH/ACN 60/8.5/31.5 (v/v/v) with 0.1% FA. The flow rate was 50 µL/min at 50 °C.*

The chromatogram in ***Figure 35*** was obtained with the commercially obtained 0.5 mm ID ACE SPH column with a MP composition consisting of H_2O/MeOH/ACN 60/8.5/31.5 (*v/v/v*) and a flow rate of 50 µL/min at 50 °C. The resolution between 22R-OHC and 25-OHC, 25-OHC and 24S-OHC, 24S-OHC and 27-OHC was R_S=2.7, R_S=1.3 and R_S=1.0, respectively. The column efficiency that was calculated for 24S-OHC was N=1700, which is 850 plates (N) less than that of the in-house packed 0.1 mm ID column. Furthermore, with the conditions used to obtain the chromatogram in ***Figure 35***, the resolution between the analytes was good enough for identification within an analysis time of 10 minutes. As a comparison, the in-house packed 0.5 mm ID columns with the same particles and with the same conditions (flow rate, temperature and MP composition) gave severe co-elution of the analytes.

The commercially obtained 0.5 mm ID ACE SPH column showed considerable more promising chromatography and performances than the in-house packed 0.5 mm ID columns with 2.5 µm ACE SPH particles. The commercially obtained 0.5 mm ID column showed better retention and repeatability compared with all three in-house packed 0.5 mm columns. The chromatographic performance for all in-house packed 0.5 mm ID columns gradually decreased and both resolution and column efficiency got worse

over time. The plot in ***Figure 36*** illustrates retention time stability on the commercially obtained 0.5 mm ID ACE SPH column with 57 injections. None of the in-house packed 0.5 mm ID column was examined for stability, but as a comparison, four injections (of two of the 0.5 mm ID packed columns, shown as red and orange line) are plotted together with the commercial 0.5 mm ID column.

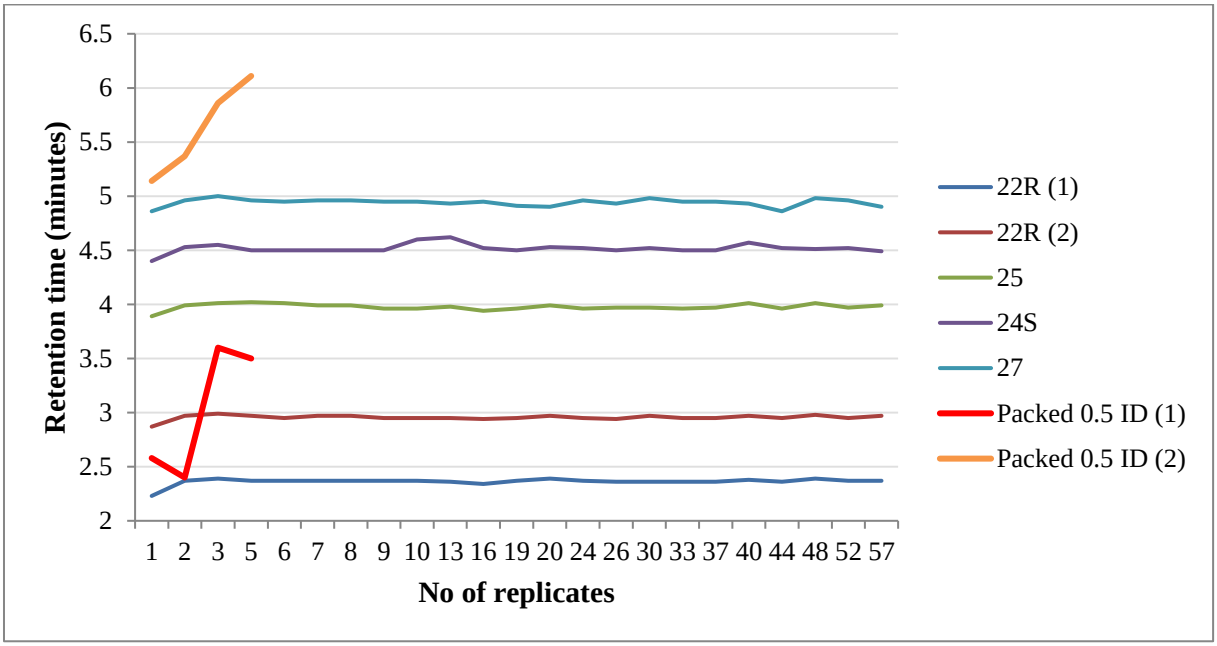


Figure 36: *The chromatographic stability with regard to retention times for the commercially obtained 0.5 mm ID ACE SPH column. The MP composition was H_2O/MeOH/ACN 60/8.5/31.5 (v/v/v) with 0.1% FA, a flow rate of 50 µL/min with isocratic elution at 50 °C. The red line are retention times for 22R-OHC and the orange line is retention times for 22S-OHC and were used to illustrate the poor stability for two of the in-house packed 0.5 mm ID columns.*

Different MP compositions, flow rates and temperatures were examined to attempt to achieve better resolution (compared to the chromatogram in ***Figure 35***). Again, several experiments were conducted, and the conditions that provided the best chromatographic performance for the commercially 0.5 mm ID column was a MP consisting of H_2O/MeOH/ACN 60/10/30 (*v/v/v*) with 0.1% FA and a flow rate of 50 µL/min at 45 °C (chromatogram shown in ***Figure 37***). With these conditions, the analysis time was approximately 20 minutes, which is a 2-fold increase compared to the conditions used to obtain the chromatogram in ***Figure 35***, but with an improved resolution.

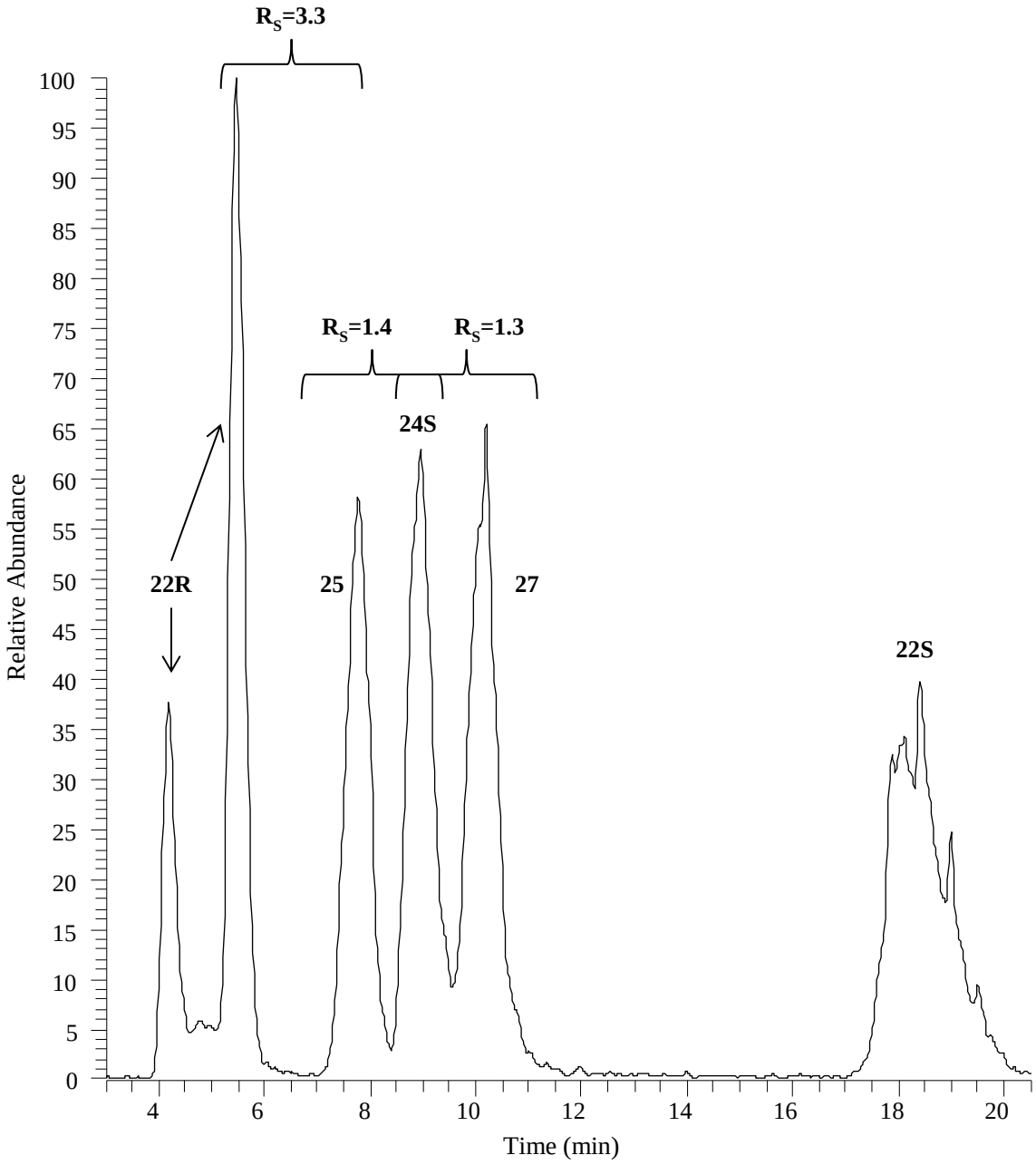


Figure 37: *A TIC chromatogram (11 points smoothing) of m/z 514.4 → 455.4 of a standard solution containing 22R-, 24S-, 25-, 27- and 22S-OHC (2.8 µg/mL each). The separation was done with a commercially obtained 0.5 mm ID ACE* SPH *column by using the system described in* ***Section 2.5.1****. The MP composition was H_2O/MeOH/ACN 60/10/30 (v/v/v) with 0.1% FA. The flow rate was 50 µL/min at 45 °C.*

The commercially obtained 0.5 mm ID ACE SPH column provided improved stability and resolution compared to the in-house packed columns with same dimension. Nevertheless, the column did not provide the analysis time and resolution needed for the determination of oxysterols in a diagnostic screening setting. As this column could provide an 18 times

theoretical increase in sensitivity compared to the 2.1 mm ID column and showed good stability, the column was investigated further with the AFFL-SPE system.

3.4 Implementing of automatic filtration and filter back-flush solid phase extraction system in the method

3.4.1 Retention factor for SPE-column to be used for on-line solid phase extraction

With the AFFL-SPE system implemented in the optimized method (***Figure 16*** *in* ***Section 1.7***), the possibility to do on-line SPE together with sample clean-up provides great advantages regarding time consuming sample preparation and higher repeatability. The analytes are loaded on the SPE column and trapped whereas excess Girard T reagents and particles and salts are flushed to waste. Analytes are trapped on the SPE column that makes it possible to inject a larger volume of sample without analytes starting to diffuse into a broad band. Analytes are eluted of the SPE column and onto the analytical column where the chromatographic separation is done.

As the analytes are trapped on the SPE column, it is essential that it is possible to elute them from the SPE column with the MP that provides the optimal separation condition on the analytical column. To make sure analytes are eluted from the SPE column and refocused on the analytical column in a small, concentrated band, it is important that the k value of analytes on the SPE column is smaller than their k value on the analytical column.

To calculate the k value, ***Equation 1*** was used. The MP composition was H_2O/MeOH/ACN 59.9/8.5/32 (v/v/v) with 0.1% FA with a flow rate of 850 µL/min at 45 °C for the analytical column while the SPE column was at ambient temperature. Two SPE columns were investigated for possible used. Each analyte was chromatographed 4 times. The k values on the Kromasil C4 and C8 SPE columns are given in ***Table 14*** together with the k values on the ACE SPH column. The k values on the C8 SPE column were larger than on the ACE SPH column. This means that analytes elutes later from the SPE column relative to the ACE SPH column under the same chromatographic conditions. This may result in band broadening on the analytical column and possibly affect the separation and thereby the resolution. However, as the C4 and C8 SPE were the available SPE columns at the time of this study, the possibility to

use either C4 or C8 SPE columns in combination with the ACE SPH column was investigated. The C4 SPE column was not able to retain the analytes. The C8 SPE column was able to retain the analytes without breakthrough, but some refocusing of the analytes were lost, leading to extra band broadening (peak width 0.33 with the AFFL-SPE and 0.28 without).

Table 14: *The calculated mean k values on the Kromasil C4 SPE column, the Kromasil C8 SPE column and the ACE SPH column.*

	22R-OHC (1)	**22R-OHC (2)**	**25-OHC**	**24S-OHC**	**27-OHC**	**22S-OHC**
C4 SPE	1.3	-	1.7	1.7	2.1	2.3
C8 SPE	4.8	-	9.3	9.8	12.9	22.9
ACE SPH	2.6	3.5	4.7	5.5	6.3	10.4

3.4.2 Optimization of automatic filtration and filter back-flush solid phase extraction system with the 0.5 mm ID ACE SuperPhenylHexyl analytical column

The conditions that provided the optimal chromatographic performance with the commercially obtained 0.5 mm ID ACE SPH column was at 50 µL/min with a MP consisting of H_2O/MeOH/ACN 60/10/30 (*v/v/v*) with 0.1% FA at 45 °C. These conditions could not be used when the AFFL-SPE system was implemented because of the increased pressure resulting in leakage in the switching system. By lowering the flow and temperature to 40 µL/min and 35 °C respectively, resolution without leakage was again obtained, shown in ***Figure 38***.

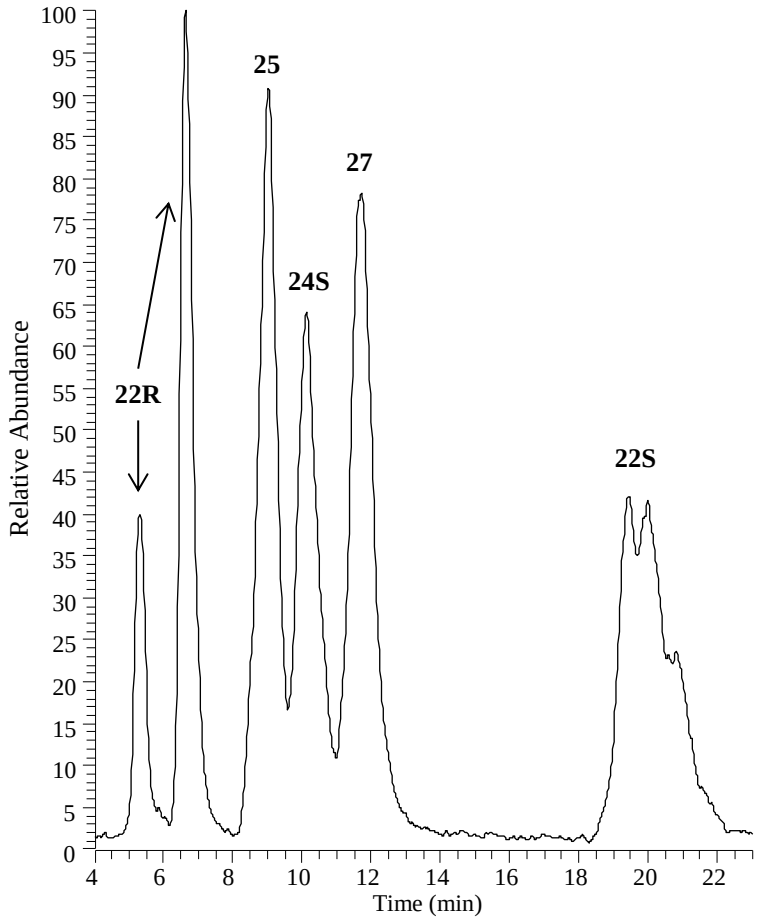


Figure 38: *A TIC chromatogram (11 points smoothing) of m/z 514.4 → 455.4 of a standard solution containing 22R-, 24S-, 25-, 27- and 22S-OHC (750 ng/mL each) separated with a commercially obtained 0.5 mm ID ACE* SPH *column with the AFFL-SPE system with a C8 SPE column by using the system described in* ***Section 2.5.1****. The MP composition was H_2O/MeOH/ACN 60/10/30 (v/v/v) with 0.1% FA. The flow rate was 40 µL/min at 35 °C. The loading pump (containing H_2O with 0.1% FA) was operated at 100 µL/min where the loading time was 1 minute.*

The implementation of the AFFL-SPE system with the commercially obtained 0.5 mm ID column did affect both the resolution and the analysis time and provided poorer chromatography (compared to analysis without the AFFL-SPE system). It was concluded to use the 2.1 mm ID ACE SPH column for analyses of biological samples as the 2.1 mm ID column provided the best resolution and analysis time needed for the determination of oxysterols in a diagnostic screening setting.

3.5 Analyses of breast cancer tumor samples and plasma samples

As mentioned, the fastest analysis with the highest resolution between the isomeric analytes was achieved with the 2.1 mm ID ACE SPH column. This column provided separation of all analytes investigated in this study within six minutes and whereas the enzymatically formed oxysterols (all except 22S-OHC) were separated within four minutes. The resolution between each oxysterol was ≥ 1.5.

Analyses of biological samples were conducted by Hanne Røberg-Larsen due to the time restriction of this study. The sample preparation is described in the appendix ***Section 6.1.3*** and ***6.1.4***. Only free oxysterols (*i.e.* not oxysterols esterified with fatty acids [136]) are detected with the sample preparation used in this study.

The analyses were performed with the ACE SPH column with serial no: A183921, as the first column was used for making microbore, capillary and nano columns (both the ACE SPH columns have the same batch no.). Nevertheless, two identical columns with the same batch no. should provide the same chromatographic performance, but it turned out that the second 2.1 mm ID ACE SPH column needed a higher amount of organic solvent in the MP to match the analysis time provided with the first 2.1 mm ID ACE SPH column. After optimization regarding the MP composition to attain similar performance, the conditions for analyzing biological samples ended up with H_2O/MeOH/ACN 57/10/33 (*v/v*) with 0.1% FA and a flow rate of 850 µL/min at 45 °C. The chromatogram for the analysis of breast cancer tumor sample (**A**) together with that of a standard solution (**B**) is shown in ***Figure 39***.

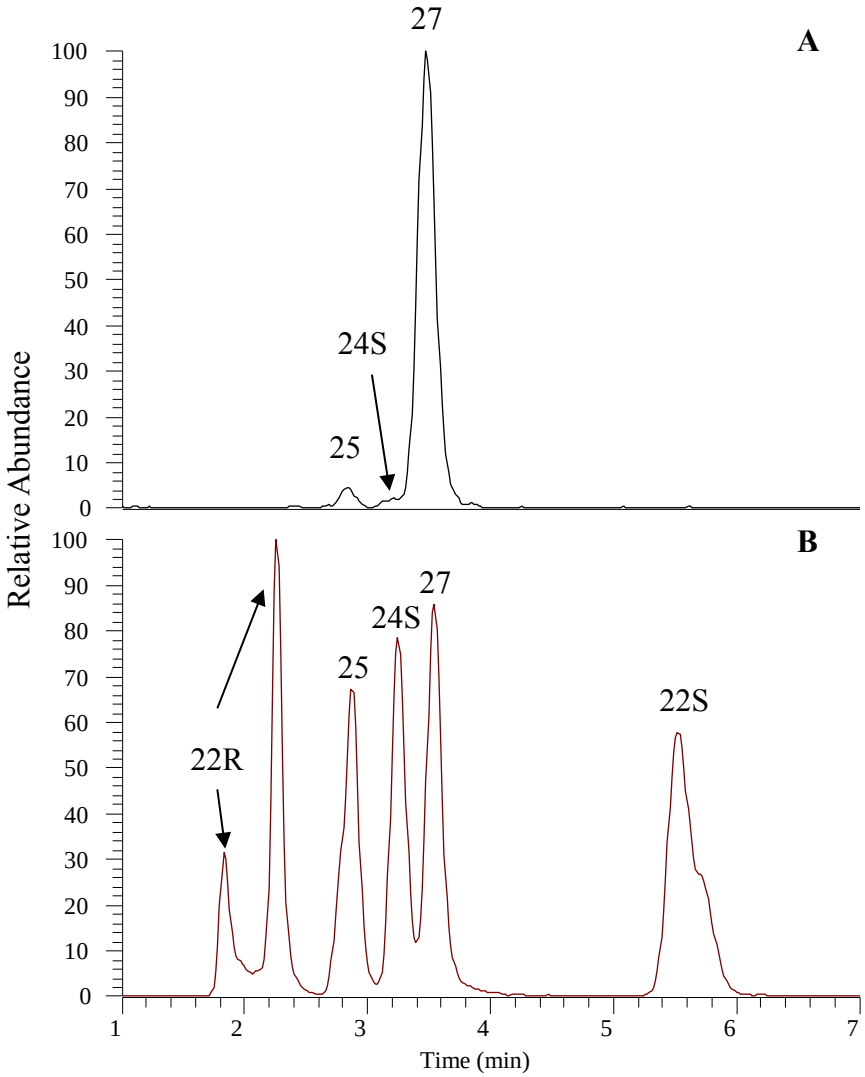


Figure 39: *TIC chromatograms (7 points smoothing) of m/z 514.4 → 455.4 of* ***A)*** *a 4.4 mg tumor sample and* ***B)*** *a standard solution containing 22R-, 24S-, 25-, 27- and 22S-OHC (2.8 µg/mL each). The separation was done with a commercially obtained 2.1 mm ID ACE SPH column by using the system described in* ***Section 2.5.1.*** *The MP consisted of H_2O/MeOH/ACN 57/10/33 (v/v/v) with 0.1% FA. The flow rate was 850 µL/min at 45 °C.*

With an injection volume of 0.7 µL of the tumor sample, it was possible to identify 25-OHC, 24S-OHC and 27-OHC by comparing the chromatograms of the standard solution with that of the tumor sample. Hence, the 2.1 mm ID column method is sensitive enough for tumor samples.

As expected concentration of oxysterols in plasma samples are in the ng/mL range [3] the method developed with the 2.1 mm ID ACE SPH column will not be sensitive enough for analyses of plasma samples. Instead, analyses of plasma samples were done by coupling the 2.1 mm ID column with the AFFL-SPE system to allow large volume injections. The implementing

the AFFL-SPE system requires that the flow rate is lowered from 850 µL/min to 700 µL/min due to the pressure limitation when introducing the SPE column. The analysis of a plasma sample is shown in ***Figure 40*.**

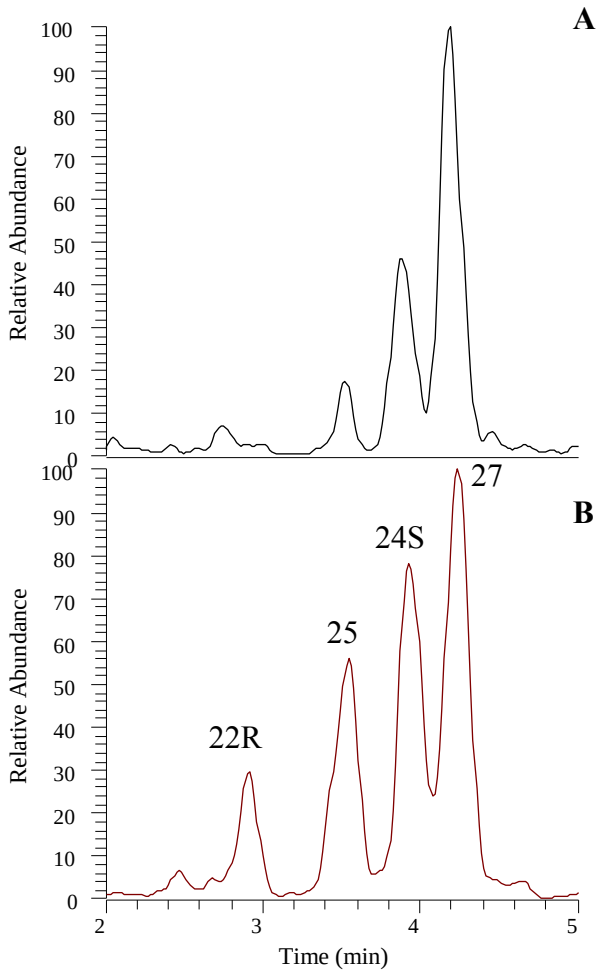


Figure 40: *TIC chromatograms (7 points smoothing) of m/z 514.4 → 455.4 of* ***A)*** *a 5 µL plasma sample and* ***B)*** *a 5 µL plasma sample spiked with 22R-, 24S-, 25- and 27-OHC (100 pg each). The separation was done with a commercially obtained 2.1 mm ID ACE* SPH *column with the AFFL-SPE system with a C8 SPE column by using the system described in* ***Section 2.5.1.*** *The MP composition was H_2O/MeOH/ACN 57/10/33 (v/v/v) with 0.1% FA. The flow rate was 700 µL/min at 55 °C.*

The chromatogram shown in ***Figure 40*** (**A**) is obtained using only 5 µL of plasma as starting material in the sample preparation. The injection volume was 60 µL for both chromatogram **A** and **B** in ***Figure 40***. The plasma sample in chromatogram **B** was spiked with 100 pg of each analyte. The intensity in chromatogram **A** and **B** was $6.36e^2$ and $1.23e^3$, respectively. The resolution between 24S-OHC and 27-OHC in chromatogram **A** in ***Figure 40*** is 1.2. A comparison of the analysis time between the method used in our group and the method developed in this study is shown in ***Figure 41***.

The determination of oxysterols in the breast cancer tumor sample was successfully completed without the need of the AFFL-SPE system (for large volume injection) because of the abundant concentration of 25-, 24S- and 27-OHC in this tumor sample. The separation was achieved within 5 minutes analysis time with acceptable resolution. The determination of oxysterols in a 5 µL plasma sample was also successfully done, but the low concentration of oxysterols in this sample emphasized the need for large volume injection using the AFFL-SPE system. Nevertheless, this shows that the 2.1 mm ID ACE SPH

column with the AFFL-SPE system can be used for the determination of oxysterols in a diagnostic screening setting.

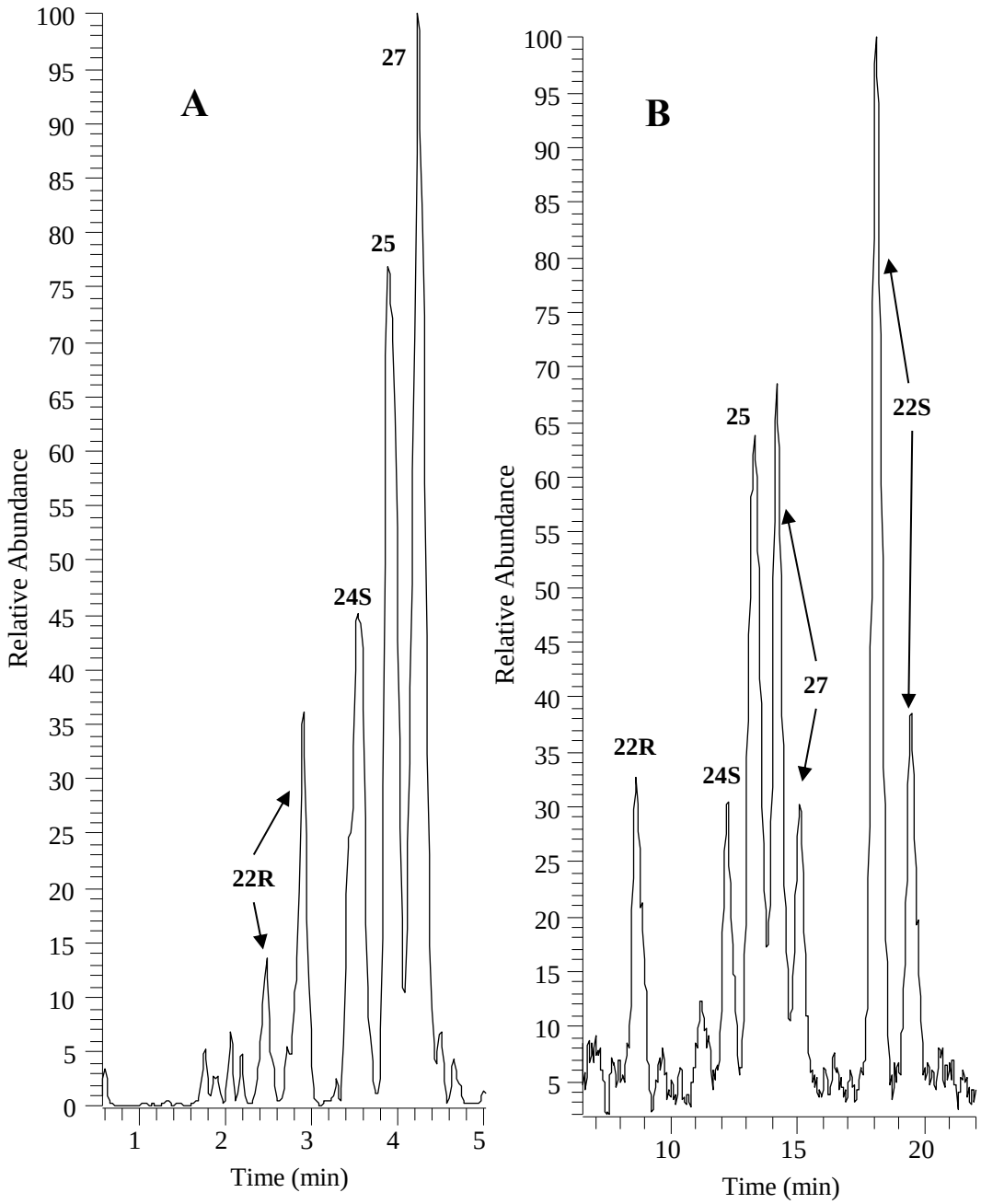


Figure 41: *TIC chromatograms (7 points smoothing) of m/z 514.4 → 455.4 of* ***A)*** *a 5 µL plasma sample spiked with 22R-, 24S-, 25- and 27-OHC (100 pg each) with 60 µL injection volume and* ***B)*** *a standard solution containing 22R-, 24S-, 25-, 27- and 22S-OHC (81 pM each) with 100 µL injection volume. The chromatogram* ***A*** *was obtained with the same conditions as used in* ***Figure 40****. The chromatogram* ***B*** *is reproduced with permission from [56].*

4 Conclusion

A method for determination the side-chained oxysterols 22R-, 24S-, 25-, 27- and 22S-OHC was developed. The separation of the enzymatically formed oxysterols was achieved within 4 minutes analysis time with a resolution > 1.5 for all. This was done by using a 2.1 mm ID analytical column from ACE with SuperPhenylHexyl SP on core-shell particles. The SP provided the selectivity needed for the separation of the oxysterols while the core-shell particles made it possible to utilize high flow rate and which was the major contribution to achieve the short analysis time.

The examination of the reversed phase columns with C18 SP did not provide the analysis time nor the resolution that was desirable for the determination of the oxysterols in a diagnostic screening setting. Nevertheless, the investigation of the Thermo Hypersil Gold column led to an improvement of the analysis time compared to previously work on the separation of side-chained oxysterols using Hypersil Gold columns.

Two columns from Waters, both with somewhat alternative SP were investigated due to their reported applicability for separation of sterols and vitamins. The Torus 1-AA column showed poor performance in LC-ESI-MS mode, whereas the Torus 2-PIC had no selectivity towards the side-chained oxysterols and which led to co-elution of them all.

Three in-house packed columns with different ID were made using the SP from the ACE SPH column. The in-house packed 0.1 mm ID column with provided the best chromatographic performance (*i.e.* highest resolution). This was not expected due to previously reported performance on in-house packed columns with core-shell particles, where the optimal chromatographic performance has been achieved using larger ID columns compared to more narrow ID columns [137]. By reducing the ID of the column from 2.1 mm (the commercially obtained ACE SPH column) to 0.1 mm, the theoretical sensitivity increased by a factor of 440. However, none of the in-house packed columns had an acceptable analysis time or resolution (> 5 minutes analysis time and R_S < 1.5).

The determination of the oxysterols in tumor samples and plasma samples from breast cancer patients using the method developed in this study were successfully performed with an analysis time of < 5 minutes and a resolution between the two closest peaks (24S- and 27-OHC) of 1.2

(for the plasma sample). The AFFL-SPE system was implemented for the analysis of the plasma sample, and which enabled the possibility for on-line sample clean up and large volume injection. By utilizing large volume injection (*e.g.* 100 µL injection), the method developed using the 2.1 mm ID ACE SPH column was sensitive enough for the determination of oxysterols in a single drop of blood.

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6 Appendix

6.1 Standard and sample preparation

6.1.1 Oxidation with cholesterol oxidase from Streptomyces sp.

The standard solution for the method development was made by transferring an appropriate aliquot (depending on the required concentration and compound) of stock solution to a safe-lock tube and evaporated it into dryness with a centrifugal evaporator. After evaporation into dryness, the residue was re-dissolved in 20 µL 2-propanol with a vortex. Aliquots of 200 µL 30 µg/mL cholesterol oxidase dissolved in 50 mM phosphate buffer pH 7 was added to the safe-lock tube to convert the 3β-hydroxy-5-ene to 3-oxo-4-ene (***Figure 4***). This reaction was initiated by heating the solution to 37 °C for 1 hour using a thermoshaker.

6.1.2 Derivatization of the oxysterols with Girard T reagent

Analytes in standard solution were charge tagged with Girard T reagent by derivatization as shown in ***Figure 5***. This was done by adding 500 µL of a mixture consisting of 15 mg Girard T reagent, 15 µL glacial acetic acid and 500 µL of MeOH to the sample. This gave 720 µL as the final volume in the safe-lock tubes where the analyte were dissolved in ≈ 70% MeOH. The derivatization reaction was carried out overnight in the dark at room temperature. The sample clean up procedure was carried out either on-line or off-line as described in ***Section 1.3.4 and 1.3.5.***

6.1.3 Preparation of plasma sample

An aliquot of 5 µL plasma was transferred to a safe-lock tube and mixed with 25 µL 1.5 nM 25-hydroxycholesterol-d_6 (25d_6-OHC as internal standard) and 5 µL 6 µM cholesterol-25, 26, 27 ^{13}C (^{13}C-cholesterol for monitoring of autoxidation) and which was vortexed and evaporated into dryness. After this step, the sample preparation was the same as described above in ***Section 6.1.1 and 6.1.2.***

6.1.4 Preparation of tumor sample

An aliquot of 4.4 mg tumor was transferred to a safe-lock tube and mixed with 500 µL 1.5 nM 25d_6-OHC and 30 µL 6 µM ^{13}C-cholesterol and vortexed. The off-line SPE was performed with an Oasis SPE column. Unlike the SPE column used for the off-line sample clean up as described in ***Section 2.2.4,*** the Oasis SPE column does not need to be conditioned before applying the sample. 200 µL pre-vortexed sample was applied to the Oasis SPE column. The analyte was eluted of the Oasis SPE column using 250 µL MeOH. The eluate was evaporated into dryness and re-dissolved in 45 µL 2-propanol. 20 µL of that solution was transferred to a new safe lock tube where 200 µL of cholesterol oxidase was applied. After this step, the sample preparation was the same as described in ***Section 6.1.1 and 6.1.2.***

6.2 Supplementary data for the Dionex UltiMate 3000 pump

When referring to "pump channels" or "channel A / B" on the pump, it refers to what kind of flask the solvent is coming from. There are four channels in this system. Throughout this study, channel A always consisted of type 1 H_2O with 0.1% FA and channel B consisted of MeOH with 0.1% FA unless otherwise stated. This means that when the MP composition is discussed in this thesis, a MP consisting of 60% MeOH will correspond to channel A supplying 40% of the MP. Channel C was only used when mixing two different organic solvents, and channel D consisted of MeOH and was used for cleaning the sample injection loop and the injection needle in between injections.

According to Thermo Scientifics home page, the pump should be able to deliver a flow rate of 0.001 mL/min to 10 mL/min, but experience indicates that the pump should not be operated below 50 µL/min due to an unstable supply of MP. The maximum pumping pressure is 620 bar. If the pressure exceeds this limit, the pump shuts down automatically.

The column oven can be adjusted by 0.1 °C and is capable of withstanding temperatures in the range of 5.0 °C to 80 °C. The oven is equipped with a fan connected to a heat/cooling block for fast temperature adjustment. This means that changing the temperature in the column oven is done quite rapidly, but the temperature has to be constant for some time to get the column in equilibrium with the column temperature.

The autosampler has room for 160 autosampler vials and is equipped with a thermostat that was set to 4 °C.

Figure S 1: *The Dionex UltiMate 3000 HPLC system from Thermo Scientific, with integrated MP reservoirs, a pump with degasser, a column oven and an autosampler.*

6.3 Interpretation of chromatogram

The chromatograms are shown with a x-axis and a y-axis, where the x-axis shows the time in minutes and the y-axis shows the intensity. The intensity is given as the relative abundance and is fixed to 100 for the base peak (the most intense peak) in the chromatogram, whereas the other peaks appear as relative to it. When discussing retention times, it means the time it takes from the sample injection to the elution of a compound and detection.

When presenting chromatograms, the term total ion current (TIC) is used. This represents the summed intensity across the entire range of masses being detected at every point in the analysis.

When using the SRM mode for the oxysterols, the only masses that are shown in a TIC chromatogram is the parent mass (*m/z* 514.4) and the fragment masses (*m/z* 455.4 and 427.4).

6.4 Mass spectrometer optimization

To establish secure identification of a compound with MS/MS, two fragment ions should be used for identification and quantification. The analytes investigated has the same molecular mass with the same fragmentation mechanism so the optimization was carried out with 25-OHC. A standard solution containing 14 µg/mL derivatized 25-OHC was directly infused into the electrospray ionization source connected to the MS using a syringe with a syringe pump that was operated with a flow rate of 3 µL/min.

For both of the MS instruments (the TSQ Vantage and the TSQ Quantiva) an automatic optimization was carried out. The data obtained from the optimization can be transferred into the instrument method. The parameters for the electrospray ionization source connected to the TSQ Vantage were selected by following the recommendations from Thermo Scientific as shown in ***Table 8.*** A MS and a MS/MS spectra of 25-OHC with Girard T as derivatization reagent is shown in ***Figure S 2***. An overview of the MS and the MS/MS *m/z* values that the MS was targeted on is shown in ***Table 9***.

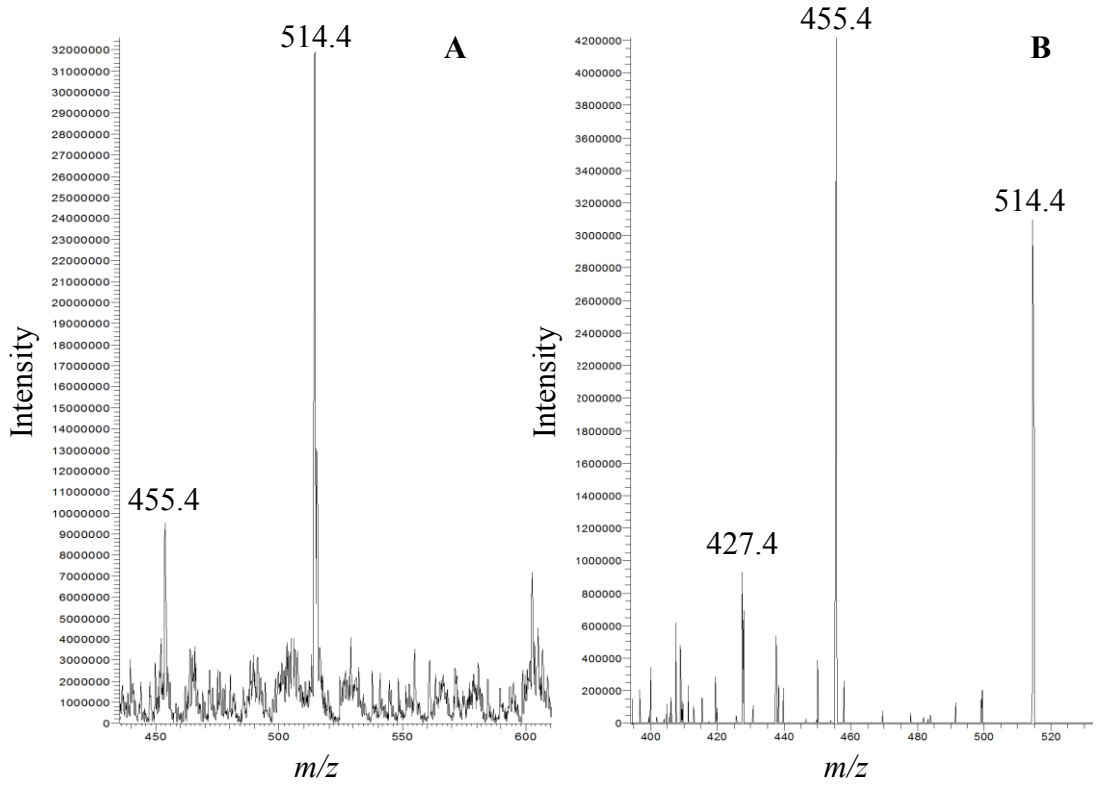

Figure S 2: A) *The MS spectrum (m/z 440-610) of 2 µL 14 µg/mL Girard T derivatized 25-OHC dissolved in 0.1% FA in 70% MeOH.* ***B)*** *The MS/MS spectrum (m/z 400-530) of the fragmentation of* m/z *514.4 to* m/z *455.4 and* m/z *427.4.*

6.5 Supplementary figures

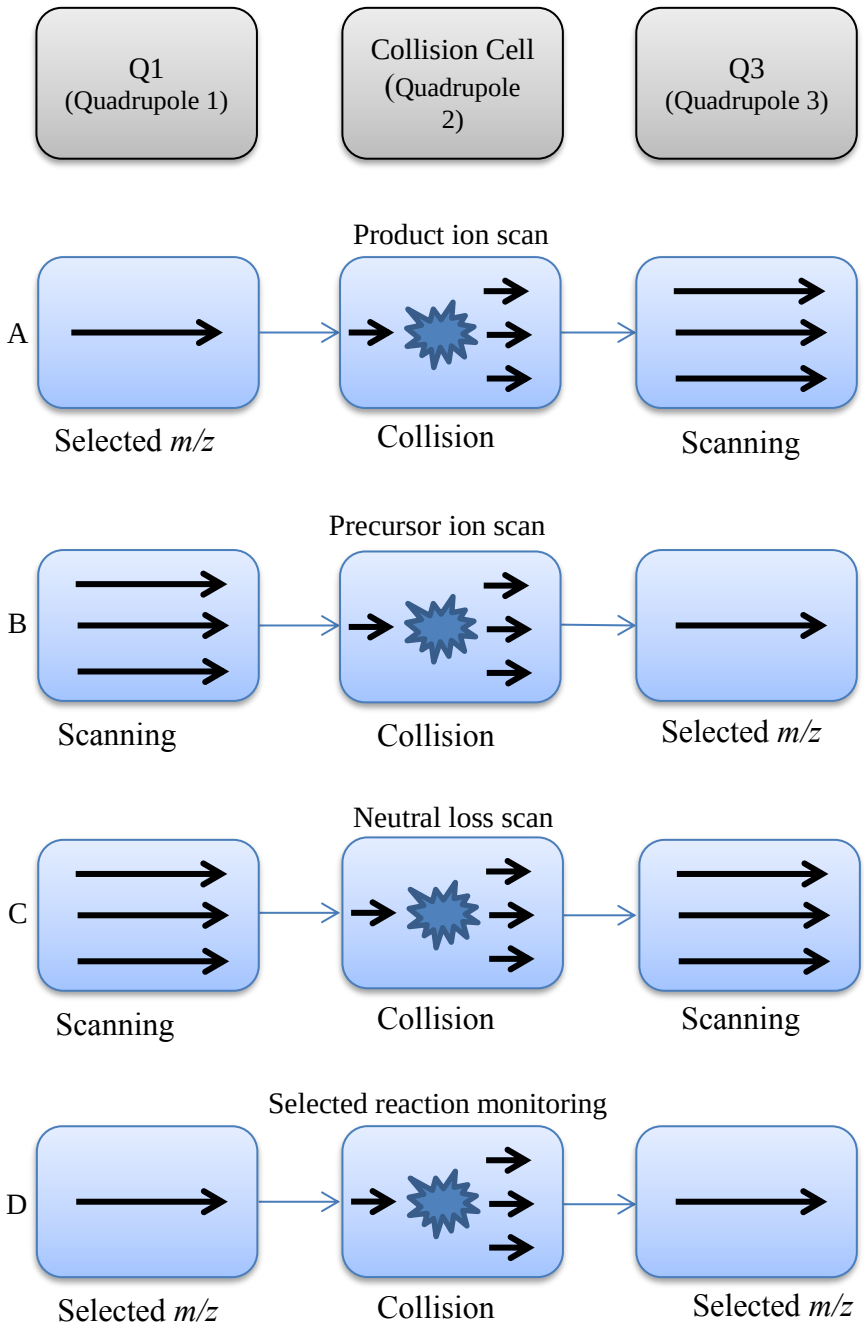

Figure S 3: *The different modes to operate the TripleQ-MS.* ***A)*** *The product ion scan where Q1 transmits selected ions with a specific m/z value, collision in Q2 and fragments are analyzed by scanning in Q3. This mode is used for structural elucidation.* ***B)*** *The precursor ion scan where Q1 scans to identify the precursor ions that gives a specific ion in Q3 upon fragmentation in Q2.* ***C)*** *The neutral loss scan where Q1 and Q3 are scanning simultaneously with a constant m/z difference to identify all parent ions that loose a specific neutral fragment (neutral fragments will not be measured due to their lack of charge).* ***D)*** *The selected reaction monitoring (SRM) for selective and sensitive detection of one analyte (or multiple reaction monitoring (MRM) for more than one analyte).*

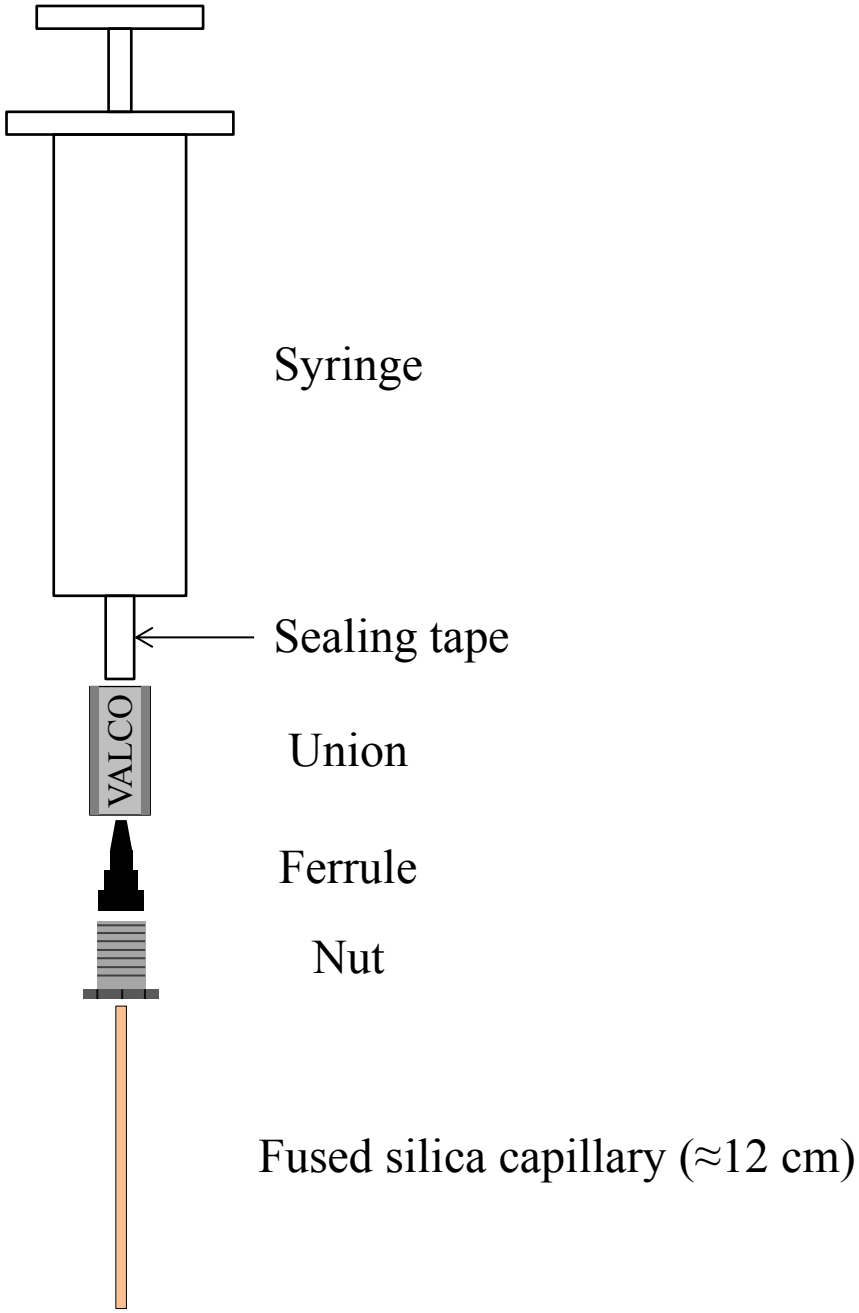

Figure S 4: *An illustration of the construction of the syringe used for transferring the slurry to the packing chamber.*

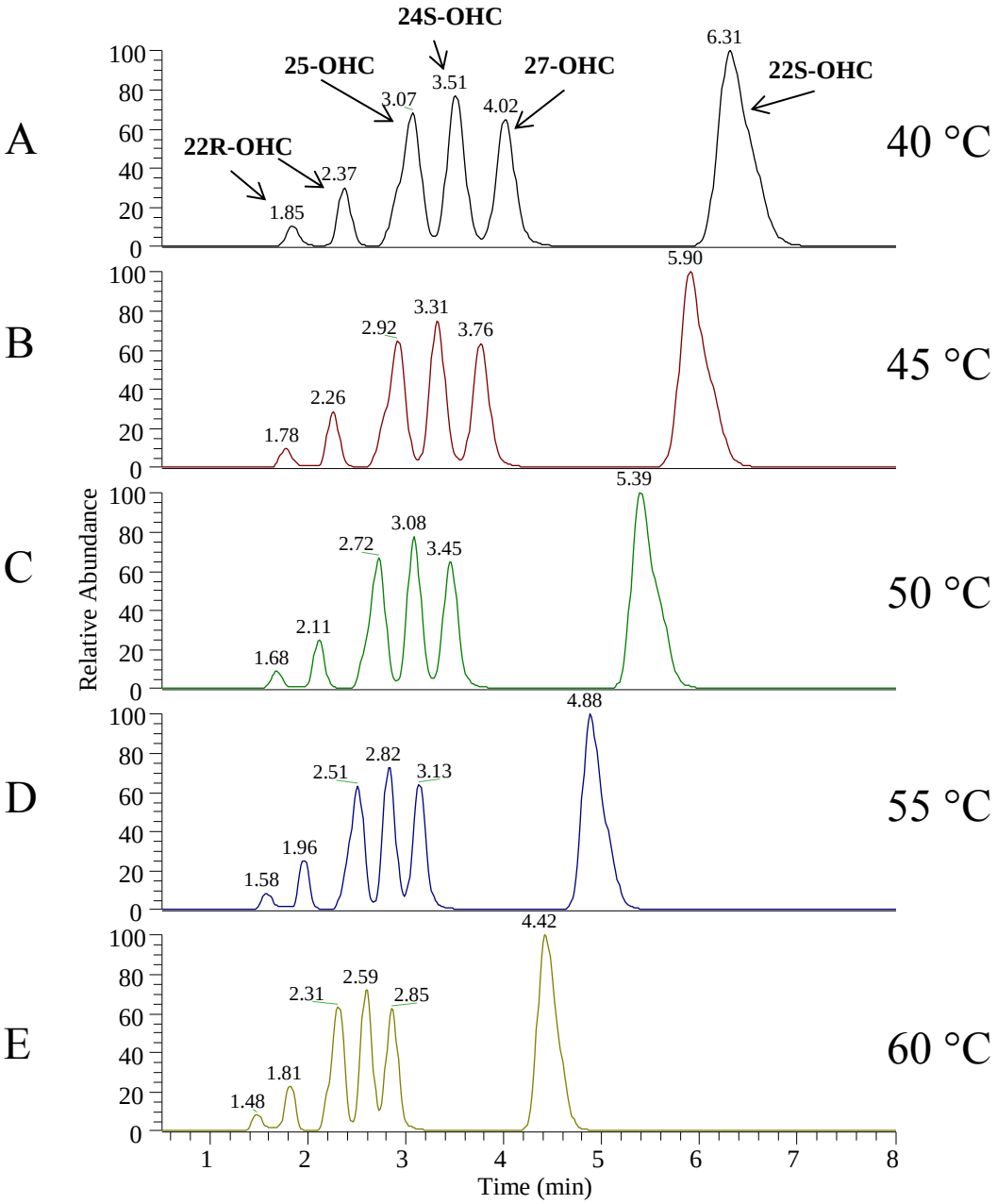

Figure S 5: *TIC chromatograms (7 points smoothing) of m/z 514.4 → 455.4 of a standard solution containing 22R-, 24S-, 25-, 27- and 22S-OHC (2.8 µg/mL each). The separation was done with the 2.1 mm ID ACE SPH column by using the system described in* ***Section 2.5.1****. The MP composition consisted of H_2O/MeOH/ACN 60.5/8/31.5 (v/v/v) with 0.1% FA. The flow rate was 800 µL/min at* ***A)*** *40 °C,* ***B)*** *45 °C,* ***C)*** *50 °C,* ***D)*** *55 °C and* ***E)*** *60 °C.*

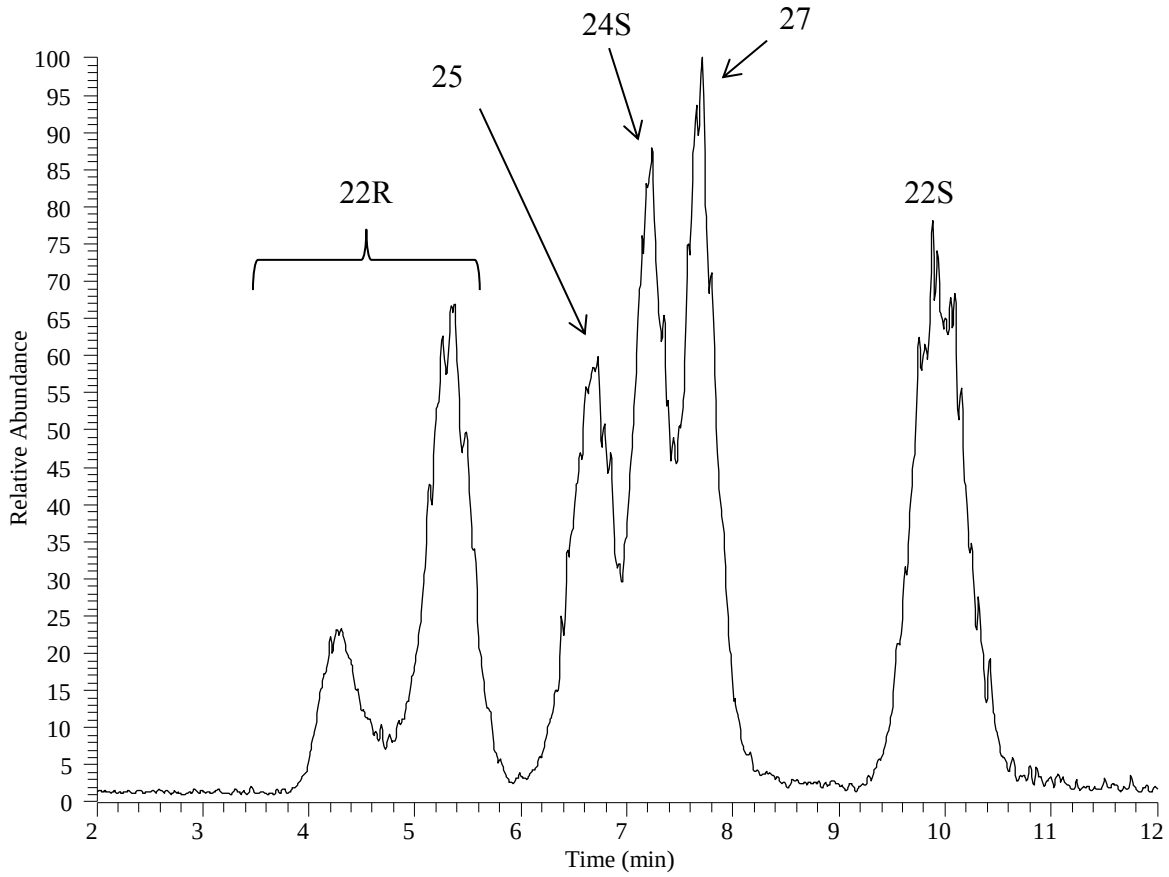

Figure S 6: *A TIC chromatogram (7 points smoothing) of m/z 514.4 → 455.4 of a standard solution containing 22R-, 24S-, 25-, 27- and 22S-OHC (2.8 µg/mL each). The separation was done with an in-house packed 0.3 mm ID column with SPH SP by using the system described in* ***Section 2.5.1****. The MP composition consisted of H_2O/MeOH/ACN 61/8/31 (v/v/v) with 0.1% FA. The flow rate was 25 µL/min at 50 °C.*

6.6 Supplementary tables

Table S 1: *A systematical investigation of the ACE UltraCore SuperC18 column with different amounts of MeOH as MP at different flow rates and temperatures.*

% MeOH	Flow	Temperature	Separation	No. of analytes detected	Resolution	Analysis time	Pressure (bar)
60	200	25	None	None	< 1.5	>20 min	245
		50	None	None	< 1.5	>20 min	165
		80	Some	22R, 24/25	< 1.5	>20 min	140
	400	25	None	None	< 1.5	>20 min	510
		50	None	None	< 1.5	>20 min	390
		80	Yes	Unknown/co-eluting	< 1.5	16 min	310
	600	25	NA	NA	NA	NA	>620
		50	NA	NA	NA	NA	>620
		80	Some	Unknown/co-eluting	< 1.5	11 min	470
70	200	25	None	None	< 1.5	>20 min	195
		50	Some	Unknown/co-eluting	< 1.5	>20 min	150
		80	Yes	Unknown/co-eluting	< 1.5	7 min	130
	400	25	None	None	< 1.5	>20 min	465
		50	Some	Unknown/co-eluting	< 1.5	12 min	360
		80	Some	Unknown/co-eluting	< 1.5	4 min	295
	600	25	NA	NA	NA	NA	>620
		50	Some	Unknown/co-eluting	< 1.5	9 min	535
		80	Some	Unknown/co-eluting	< 1.5	3 min	390
80	200	25	Some	Unknown/co-eluting	< 1.5	17 min	170
		50	Yes	Unknown/co-eluting	< 1.5	7 min	125
		80	Some	Unknown/co-eluting	< 1.5	4 min	95
	400	25	Some	Unknown/co-eluting	< 1.5	8 min	400
		50	Some	Unknown/co-eluting	< 1.5	3 min	300
		80	None	1 peak	< 1.5	2 min	215
	600	25	NA	NA	NA	NA	>620
		50	Some	Unknown/co-eluting	< 1.5	3 min	500
		80	None	1 peak	< 1.5	2 min	345

***Table S 2**: Some of the different conditions that was investigated for the ACE Super C18 column. B in this table is consisting of MeOH with 0.1% FA.*

Flow rate µL/min	Temperature °C	Isocratic elution (% B)	Resolution	Analysis time (minutes)	Effect / comments
200	22	70	≪ 1.5	> 15	Analysis time too long.
300	22	85	≪ 1.5	3	Resolution between 25-, 24S- and 27-OHC is not acceptable.
350	22	80	≪ 1.5	5	Resolution between 25-, 24S- and 27-OHC is not acceptable.
400	22	75	≪ 1.5	8	Resolution between 25-, 24S- and 27-OHC is not acceptable.
400	22	77	≪ 1.5	8	Resolution between 25-, 24S- and 27-OHC is not acceptable.
450	30	70	< 1.5	> 10	Analysis time too long.
490	35	68	< 1.5	> 12	Analysis time too long.
530	40	68	< 1.5	15	Resolution between 25-, 24S- and 27-OHC is not acceptable.
560	45	65	< 1.5	> 15	Analysis time too long.
600	55	60	≪ 1.5	> 17	Resolution not acceptable and analysis time too long.
600	50	63	≪ 1.5	> 17	Resolution not acceptable and analysis time too long.
600	50	65	< 1.5	14	Resolution between 25-, 24S- and 27-OHC is not acceptable.
650	60	60	≪ 1.5	> 17	Resolution not acceptable and analysis time too long.
650	60	65	≪ 1.5	7	Resolution between 25-, 24S- and 27-OHC is not acceptable.
650	65	65	≪ 1.5	6	Resolution between 25-, 24S- and 27-OHC is not acceptable.
700	65	70	≪ 1.5	4.5	Resolution between 25-, 24S- and 27-OHC is not acceptable.
200	15	80	≪ 1.5	> 12	Resolution not acceptable and analysis time too long.
250	15	80	≪ 1.5	8	Resolution between 25-, 24S- and 27-OHC is not acceptable.
300	15	80	≪ 1.5	7	Resolution between 25-, 24S- and 27-OHC is not acceptable.
330	25	70	< 1.5	> 15	Analysis time too long.
330	15	75	≪ 1.5	> 15	Resolution not acceptable and analysis time too long.
350	25	65	≪ 1.5	≫ 15	Analysis time too long.
350	20	72	≪ 1.5	> 15	Resolution not acceptable and analysis time too long.
350	20	75	≪ 1.5	11	Resolution between 25-, 24S- and 27-OHC is not acceptable.
355	20	65	≪ 1.5	≫ 15	Analysis time too long.
370	20	68	≪ 1.5	≫ 15	Resolution not acceptable and analysis time too long.
380	15	75	≪ 1.5	12	Resolution between 25-, 24S- and 27-OHC is not acceptable.
100	25	80	≪ 1.5	> 15	Resolution between 25-, 24S- and 27-OHC is not acceptable.
150	25	80	≪ 1.5	> 15	Resolution between 25-, 24S- and 27-OHC is not acceptable.

Table S 3: *The different mixtures of MeOH and ACN, flow rates and temperature that were investigated for the ACE SuperC18 column.*

Flow (µL/min)	Temp (°C)	Amount H_2O (A)	Amount MeOH (B)	Amount ACN (C)	Resolution
550	25	40	30	30	< 1.5
450	25	40	30	30	< 1.5
350	25	40	20	40	< 1.5
350	25	30	40	30	< 1.5
350	25	40	30	30	< 1.5
450	25	38	30	32	< 1.5
400	30	27	68	5	< 1.5
400	30	25	70	5	< 1.5
400	30	32	67	1	< 1.5
400	30	31	67	2	< 1.5
400	30	30	67	3	< 1.5
400	30	31	68	1	< 1.5
400	30	30	68	2	< 1.5
400	30	29	68	3	< 1.5
400	30	30	69	1	< 1.5
400	30	29	69	2	< 1.5
400	30	28	69	3	< 1.5
400	30	29	70	1	< 1.5
400	30	28	70	2	< 1.5
400	30	27	70	3	< 1.5
420	30	32	67	1	< 1.5
420	30	31	67	2	< 1.5
420	30	30	67	3	< 1.5
420	30	31	68	1	< 1.5
420	30	30	68	2	< 1.5
420	30	29	68	3	< 1.5
420	30	30	69	1	< 1.5
420	30	29	69	2	< 1.5
420	30	28	69	3	< 1.5
420	30	29	70	1	< 1.5
420	30	28	70	2	< 1.5
420	30	27	70	3	< 1.5
440	30	32	67	1	< 1.5
440	30	31	67	2	< 1.5
440	30	30	67	3	< 1.5
440	30	31	68	1	< 1.5
440	30	30	68	2	< 1.5
440	30	29	68	3	< 1.5
440	30	30	69	1	< 1.5
440	30	29	69	2	< 1.5
440	30	28	69	3	< 1.5
440	30	29	70	1	< 1.5
440	30	28	70	2	< 1.5
440	30	27	70	3	< 1.5

Table S 4: *A systematical investigation of the ACE UltraCore SPH column with different amounts of MeOH as MP at different flow rates and temperatures.*

% MeOH	Flow	Temperature	Separation	No. of analytes detected	Resolution	Analysis time	Pressure (bar)
60	200	25	None	None	< 1.5	>20 min	168
		50	None	None	< 1.5	>20 min	175
		80	Some	Unknown/co-eluting	< 1.5	14 min	110
	400	25	None	None	< 1.5	>20 min	400
		50	Some	Unknown/co-eluting	< 1.5	>20 min	300
		80	Some	Unknown/co-eluting	< 1.5	6 min	290
	600	25	NA	NA	NA	NA	>620
		50	Some	Unknown/co-eluting	< 1.5	17 min	460
		80	Some	Unknown/co-eluting	< 1.5	5 min	445
70	200	25	Some	Unknown/co-eluting	< 1.5	>20 min	150
		50	Some	Unknown/co-eluting	< 1.5	11 min	112
		80	None	Unknown/co-eluting	< 1.5	5 min	120
	400	25	Some	Unknown/co-eluting	< 1.5	12 min	375
		50	Some	Unknown/co-eluting	< 1.5	5 min	265
		80	None	Unknown/co-eluting	< 1.5	3 min	245
	600	25	NA	NA	NA	NA	>620
		50	Some	Unknown/co-eluting	< 1.5	4 min	445
		80	None	1 peak	< 1.5	2 min	400
80	200	25	Some	Unknown/co-eluting	< 1.5	7 min	130
		50	None	1 peak	< 1.5	5 min	100
		80	None	1 peak	< 1.5	5 min	90
	400	25	None	1 peak	< 1.5	3 min	360
		50	None	1 peak	< 1.5	3 min	240
		80	None	1 peak	< 1.5	2 min	340
	600	25	NA	NA	NA	NA	>620
		50	None	1 peak	< 1.5	2 min	400
		80	NA	NA	NA	NA	NA

Table S 5: *An investigation of different gradient elutions for the commercially obtained 2.1 mm ID ACE SPH column. The MP A consisted of MeOH/H_2O 5/95 (v/v) and the MP B consisted of MeOH/ACN 10/90 (v/v), both with 0.1% FA.*

Flow rate µL/min	Temperature °C	Gradient (% B)	Gradient time	Resolution	Analysis time (minutes)	Effect / comments
500	30	42 – 50 – 68	42 – 50 in 3 minutes	$\ll 1.5$	3	Co-elution of 24S- and 27-OHC.
500	30	35 – 42 – 70	35 – 42 in 3 minutes	$\ll 1.5$	< 1	Co-elution of all analyte in one peak. It is not known why this gradient elute analytes faster than e.g. the one above.
500	30	40 – 48 – 70	40 – 48 in 3 minutes	$\ll 1.5$	4	Co-elution of 24S- and 27-OHC.
500	30	36 – 40	4 minutes	≈ 1.0	7.5	All analytes separated, poorest resolution between 24S- and 27-OHC.
500	30	37 – 42	4 minutes	< 1.5	5.5	All analytes separated, poorest resolution between 24S- and 27-OHC.

Table S 6: *An investigation of different isocratic elutions for the commercially obtained 2.1 mm ID ACE SPH column. The MP A and B composition was the same as in* ***Table S 5****.*

Flow rate µL/min	Temperature °C	Isocratic elution (% B)	Resolution	Analysis time (minutes)	Effect / comments
500	30	42	$\ll 1.5$	4	Cu-elution of 24S- and 27-OHC.
500	30	40	$\ll 1.5$	5	Cu-elution of 24S- and 27-OHC.
500	30	38	< 1.5	8	Resolution between 25-, 24S- and 27-OHC is not acceptable.
500	30	36	< 1.5	7.5	Resolution between 25-, 24S- and 27-OHC is not acceptable.
500	30	37	≈ 1.0	9	Analysis time too long.
300	30	43	$\ll 1.5$	4	Co-elution of 22R-, 25-, 24S- and 27-OHC.
350	30	43	$\ll 1.5$	4	Co-elution of 22R-, 25-, 24S- and 27-OHC.
400	35	35	≈ 1.5	15	Analysis time too long.
400	25	38	$\ll 1.5$	9	Co-elution of 22R-, 25-, 24S- and 27-OHC.
400	30	40	$\ll 1.5$	5.5	Co-elution of 22R-, 25-, 24S- and 27-OHC.
420	25	42	$\ll 1.5$	4	Co-elution of 22R-, 25-, 24S- and 27-OHC.
430	30	39	$\ll 1.5$	6	Co-elution of 22R-, 25-, 24S- and 27-OHC.
440	25	40	$\ll 1.5$	5.5	Co-elution of 22R-, 25-, 24S- and 27-OHC.
440	30	40	$\ll 1.5$	5	Co-elution of 22R-, 25-, 24S- and 27-OHC.
450	35	35	≈ 1.5	14	Analysis time too long.
450	30	37	< 1.5	9	Resolution between 25-, 24S- and 27-OHC is not acceptable.
450	25	38	$\ll 1.5$	8	Co-elution of 22R-, 25-, 24S- and 27-OHC.
450	30	38	$\ll 1.5$	7	Co-elution of 22R-, 25-, 24S- and 27-OHC.
450	25	42	$\ll 1.5$	4	Co-elution of 22R-, 25-, 24S- and 27-OHC.
460	30	39	$\ll 1.5$	6	Co-elution of 22R-, 25-, 24S- and 27-OHC.
480	30	37	$\ll 1.5$	8.5	Co-elution of 22R-, 25-, 24S- and 27-OHC.
480	25	38	$\ll 1.5$	8	Co-elution of 22R-, 25-, 24S- and 27-OHC.
480	30	38	$\ll 1.5$	6.5	Co-elution of 22R-, 25-, 24S- and 27-OHC.
480	25	40	$\ll 1.5$	5	Co-elution of 22R-, 25-, 24S- and 27-OHC.
500	35	35	≈ 1.5	12.5	Good resolution between all analytes, too long analysis time.
500	30	37	≈ 1.0	8	Not good enough resolution.
550	30	37	< 1.5	7.5	Co-elution of 24S- and 27-OHC.
200	30	39	< 1.5	12	Co-elution of 24S- and 27-OHC.
300	30	39	< 1.5	8	Co-elution of 24S- and 27-OHC.
500	40	35	≈ 1.5	11.5	Good resolution between all analytes, too long analysis time.
700	35	35	≈ 1.5	10	Good resolution between all analytes, too long analysis time.
800	35	35	≈ 1.5	9 minutes	Good resolution between all analytes, too long analysis time.

Table S 7: *An investigation of different isocratic elutions for the commercially obtained 2.1 mm ID ACE SPH column (continued from* ***Table S 6****).*

Flow rate µL/min	Temperature °C	Isocratic elution (% B)	Resolution	Analysis time (minutes)	Effect / comments
800	45	35	≈ 1.5	7.5	Good resolution between all analytes with acceptable analysis time.
870	40	35	≈ 1.5	7.5	Good resolution between all analytes with acceptable analysis time.
500	45	35	≈ 1.5	10.5	Good resolution between all analytes, too long analysis time.
600	40	35	≈ 1.5	10	Good resolution between all analytes, too long analysis time.
700	40	35	≈ 1.5	9	Good resolution between all analytes, too long analysis time.
700	45	35	≈ 1.5	8	Good resolution between all analytes with acceptable analysis time.
800	40	35	≈ 1.5	8	Good resolution between all analytes with acceptable analysis time.
800	45	35	≈ 1.5	7.5	Good resolution between all analytes with acceptable analysis time.
500	50	35	≈ 1.5	9.5	Good resolution between all analytes, too long analysis time.
500	55	35	≈ 1.5	8.5	Good resolution between all analytes, too long analysis time.
500	60	35	< 1.5	7.5	Resolution between 24S- and 27-OHC is <1.5.
700	50	35	≈ 1.5	7.5	Good resolution between all analytes with acceptable analysis time.
700	55	35	< 1.5	6.5	Resolution between 24S- and 27-OHC is <1.5.
700	60	35	< 1.5	6	Resolution between 24S- and 27-OHC is <1.5.
800	50	35	≈ 1.5	7	Good resolution between all analytes with acceptable analysis time.
800	55	35	< 1.5	6	Resolution between 24S- and 27-OHC is <1.5.
800	60	35	< 1.5	5.5	Resolution between 24S- and 27-OHC is <1.5.

Table S 8: *A systematical investigation of the commercially obtained 2.1 mm ID ACE SPH column.* ***1)*** *The amount of H_2O in the MP was investigated while keeping the amount of MeOH and ACN constant.* ***2)*** *The amount of MeOH in the MP was investigated while keeping the amount of H_2O and ACN constant. The flow rate was 800 µL/min at 50 °C for both tables.*

1

	H2O	MeOH	ACN									
	A	B	C	Seperation	Resolution	Total analysis time	Rt 22R (1)	Rt 22R (1)	Rt 25	Rt 24S	Rt 27	Rt 22S
1	62	6.8	31.2	Yes	Good	7.4 min	1.91	2.44	3.15	3.6	4.06	6.43
2	63	6.8	30.2	Yes	Good	9.2 min	2.39	3.08	4.06	4.63	5.28	8.37
3	64	6.8	29.2	Yes	Good	> 10 min	2.97	3.89	5.23	5.97	6.89	>10
4	62	6.5	31.5	Yes	Good	7 min	1.9	2.42	3.12	3.54	3.99	6.3
5	63	5.5	31.5	Yes	Good	7.8 min	2.08	2.65	3.41	3.92	4.4	6.99
6	64	4.5	31.5	Yes	Good	8.8 min	2.29	2.92	3.78	4.34	4.9	7.8
1	61	6.8	32.2	Yes	Neutral	5.8 min	1.6	2.01	2.56	2.9	3.25	5.08
2	60	6.8	33.2	Yes	Bad	4.8 min	1.37	1.68	2.09	2.39	2.62	4.07
3	59	6.8	34.2	Yes	Bad	3.8 min	1.15	1.42	1.71	1.96	2.13	3.26
4	61	7.5	31.5	Yes	Good	6.6 min	1.75	2.21	2.84	3.23	3.63	5.67
5	60	8.5	31.5	Yes	Neutral	6.0 min	1.6	2.01	2.6	2.93	3.28	5.13
6	59	9.5	31.5	Yes	Neutral	5.4 min	1.47	1.85	2.36	2.65	2.97	4.62

2

	H2O	MeOH	ACN									
	A	B	C	Seperation	Resolution	Total analysis time	Rt 22R (1)	Rt 22R (1)	Rt 25	Rt 24S	Rt 27	Rt 22S
1	61.7	7	31.3	Yes	Good	7.2 min	1.9	2.39	3.1	3.53	3.97	6.26
2	61.7	8	30.3	Yes	Good	8.2 min	2.11	2.7	3.58	4.06	4.6	7.27
3	61.7	9	29.3	Yes	Good	9.4 min	2.36	3.05	4.09	4.62	5.31	8.37
4	61.5	7	31.5	Yes	Good	7.0 min	1.83	2.32	3	3.41	3.83	6.03
5	60.5	8	31.5	Yes	Good	6.4 min	1.68	2.11	2.72	3.08	3.45	5.39
6	59.5	9	31.5	Yes	Neutral	5.5 min	1.53	1.93	2.47	2.79	3.12	4.85
1	61.7	6	32.3	Yes	Neutral	6.4 min	1.7	2.14	2.72	3.1	3.46	5.44
2	61.7	5	33.3	Yes	Bad	5.5 min	1.53	1.91	2.37	2.74	3.02	4.72
3	61.7	4	34.3	Yes	Bad	5.0 min	1.38	1.71	2.09	2.41	2.62	4.11
4	62.5	6	31.5	Yes	Good	7.5 min	2.01	2.55	3.3	3.76	4.24	6.69
5	63.5	5	31.5	Yes	Good	8.5 min	2.19	2.8	3.61	4.15	4.67	7.42
6	64.5	4	31.5	Yes	Good	9.5 min	2.39	3.08	3.96	4.57	5.16	8.24

Table S 9: *A systematical investigation of the ACE commercially obtained SPH column, continued from* ***Table S 8****. 3)* *The amount of ACN in the MP was investigated while keeping the amount of MeOH and H_2O constant. The flow rate was 800 µL/min at 50 °C.*

	H2O	MeOH	ACN									
	A	B	C	Seperation	Resolution	Total analysis time	Rt 22R (1)	Rt 22R (1)	Rt 25	Rt 24S	Rt 27	Rt 22S
1	61.7	6.3	32	Yes	Neutral	6.5 min	1.76	2.21	2.82	3.21	3.59	5.65
2	61.7	5.3	33	Yes	Bad	5.8 min	1.58	1.98	2.49	2.84	3.13	4.93
3	61.7	4.3	34	Yes	Bad	5.0 min	1.43	1.78	2.18	2.51	2.74	4.29
4	61.2	6.8	32	Yes	Neutral	6.5 min	1.68	2.11	2.7	3.07	3.43	5.37
5	60.2	6.8	33	Yes	Bad	5.0 min	1.42	1.75	2.19	2.49	2.75	4.27
6	59.2	6.8	34	Yes	Bad	4.0 min	1.17	1.45	1.78	2.01	2.21	3.4
1	61.7	7.3	31	Yes	Good	7.5 min	1.95	2.49	3.25	3.68	4.15	6.56
2	61.7	8.3	30	Yes	Good	8.5 min	2.18	2.8	3.71	4.2	4.78	7.57
3	61.7	9.3	29	Yes	Good	10 min	2.44	3.17	4.27	4.81	5.56	8.77
4	62.2	6.8	31	Yes	Good	8.0 min	2.06	2.64	3.43	3.89	4.4	6.96
5	63.2	6.8	30	Yes	Good	10 min	2.52	3.28	4.35	4.96	5.67	9
6	64.2	6.8	29	Yes	Good	> 10 min	3.15	4.14	5.6	6.38	7.4	> 10

Good	Less than 5% overlap between the closest peaks.
Neutral	Less than 10% overlap between the closest peaks.
Bad	More than 10% overlap between the closest peaks.

3

Table S 10: *An investigation of the in-house packed 0.1 mm ID column. The MP A consisted of MeOH/H_2O 5/95 (v/v) and the MP B consisted of MeOH/ACN 10/90 (v/v), both with 0.1% FA*

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Flow rate µL/min	Temperature °C	Unknown gradient elution (% B)	Resolution	Analysis time (minutes)	Effect / comments
300	20	31	≪ 1.5	> 20	Analysis time too long.
300	20	33	≪ 1.5	16	Analysis time too long.
300	20	35	≪ 1.5	12	Co-elution of 22R-, 25-, 24S- and 27-OHC.
300	20	36	≪ 1.5	11	Co-elution of 22R-, 25-, 24S- and 27-OHC.
300	20	38	≪ 1.5	8.5	Co-elution of 22R-, 25-, 24S- and 27-OHC.
300	20	40	≪ 1.5	7	Co-elution of 22R-, 25-, 24S- and 27-OHC.
350	20	31	≪ 1.5	> 20	Analysis time too long.
350	20	33	≪ 1.5	15	Co-elution of 22R-, 25-, 24S- and 27-OHC.
350	20	35	≪ 1.5	11	Co-elution of 22R-, 25-, 24S- and 27-OHC.
350	20	36	≪ 1.5	9.5	Co-elution of 22R-, 25-, 24S- and 27-OHC.
350	20	38	≪ 1.5	8	Co-elution of 22R-, 25-, 24S- and 27-OHC.
350	20	40	≪ 1.5	6	Co-elution of 22R-, 25-, 24S- and 27-OHC.
400	20	36	≪ 1.5	8.5	Co-elution of 22R-, 25-, 24S- and 27-OHC.
400	20	38	≪ 1.5	7	Co-elution of 22R-, 25-, 24S- and 27-OHC.
400	20	40	≪ 1.5	5.5	Co-elution of 22R-, 25-, 24S- and 27-OHC.
450	20	31	≪ 1.5	17	Co-elution of 22R-, 25-, 24S- and 27-OHC.
450	20	33	≪ 1.5	11	Co-elution of 22R-, 25-, 24S- and 27-OHC.
450	20	35	≪ 1.5	9	Co-elution of 22R-, 25-, 24S- and 27-OHC.
500	20	27	≪ 1.5	> 20	Analysis time too long.
500	20	29	≪ 1.5	> 20	Analysis time too long.
500	20	31	≪ 1.5	15	Co-elution of 22R-, 25-, 24S- and 27-OHC.
500	20	33	≪ 1.5	10.5	Co-elution of 22R-, 25-, 24S- and 27-OHC.
500	20	35	≪ 1.5	8.5	Co-elution of 22R-, 25-, 24S- and 27-OHC.
750	20	27	≪ 1.5	> 20	Analysis time too long.
750	20	28	< 1.5	> 20	Analysis time too long.
750	50	28	< 1.5	17	Co-elution of 24S- and 27-OHC.
750	50	29	< 1.5	14.5	Co-elution of 24S- and 27-OHC.
900	50	28	< 1.5	14.5	Co-elution of 24S- and 27-OHC.
900	50	30	< 1.5	10	Co-elution of 24S- and 27-OHC.
900	50	33	< 1.5	6	Co-elution of 22R-, 25-, 24S- and 27-OHC.

Table S 11: *An investigation of the in-house packed 0.1 mm ID column, continued from* ***Table S 10****.*

Flow rate µL/min	Temperature °C	Unknown gradient elution (% B)	Resolution	Analysis time (minutes)	Effect / comments
1000	50	27	≪ 1.5	16	Co-elution of 24S- and 27-OHC.
1000	50	28	≪ 1.5	12.5	Co-elution of 24S- and 27-OHC.
1000	50	29	≪ 1.5	10	Co-elution of 24S- and 27-OHC.
1000	50	30	≪ 1.5	8.5	Co-elution of 24S- and 27-OHC.
1100	50	27	< 1.5	14.5	Resolution between 25-, 24S- and 27-OHC is not acceptable.
1100	50	28	≪ 1.5	12	Co-elution of 24S- and 27-OHC.
1100	50	29	≪ 1.5	9.5	Co-elution of 24S- and 27-OHC.
1100	50	30	≪ 1.5	8	Co-elution of 24S- and 27-OHC.
1100	65	22	≈ 1.0	> 20	Analysis time too long.
1100	65	23	≈ 1.0	> 20	Analysis time too long.
1100	65	24	< 1.5	> 20	Analysis time too long.
1100	65	25	≪ 1.5	15.5	Co-elution of 25-, 24S- and 27-OHC.
1100	65	26	≪ 1.5	12.5	Co-elution of 22R-, 25-, 24S- and 27-OHC.
1100	65	27	≪ 1.5	10	Co-elution of 22R-, 25-, 24S- and 27-OHC.
1300	65	22	-	-	Leakage in LC system.
1500	65	22	-	-	Leakage in LC system.
1700	65	22	-	-	Leakage in LC system.
1800	65	22	-	-	Leakage in LC system.

Table S 12: *An investigation of the in-house packed 0.1 mm ID column. The MP A consisted of MeOH/H_2O 5/95 (v/v) and the MP B consisted of MeOH/ACN 20/80 (v/v), both with 0.1% FA.*

Flow rate µL/min	Temperature °C	Unknown gradient elution (% B)	Resolution	Analysis time (minutes)	Effect / comments
1500	65	25	-	-	Leakage in LC system.
1400	65	25	≈ 1.0	> 20	Suspected leakage in LC system, analysis time too long.
1400	65	23	-	-	Leakage in LC system.
1100	65	32	≪ 1.5	7.5	Co-elution of 22R-, 25-, 24S- and 27-OHC.
1100	65	27	< 1.5	16	Co-elution of 22R-, 25-, 24S- and 27-OHC.
700	55	36	≪ 1.5	6	Co-elution of 22R-, 25-, 24S- and 27-OHC.
800	55	32	≪ 1.5	9.5	Co-elution of 25-, 24S- and 27-OHC.
800	55	35	≪ 1.5	6.5	Co-elution of 25-, 24S- and 27-OHC.
900	55	33	≪ 1.5	6	Co-elution of 25-, 24S- and 27-OHC.
900	55	34	≪ 1.5	6.5	Co-elution of 22R-, 25-, 24S- and 27-OHC.
900	55	35	≪ 1.5	6	Co-elution of 22R-, 25-, 24S- and 27-OHC.
900	55	36	≪ 1.5	5	Co-elution of 22R-, 25-, 24S- and 27-OHC.
1000	55	28	≈ 1.0	17	Resolution between 25-, 24S- and 27-OHC is not acceptable.
1000	55	30	< 1.5	6	Resolution between 25-, 24S- and 27-OHC is not acceptable.
1000	55	32	≪ 1.5	8	Co-elution of 25-, 24S- and 27-OHC.
1000	55	33	≪ 1.5	7	Co-elution of 25-, 24S- and 27-OHC.
1200	55	27	< 1.5	18	Resolution between 25-, 24S- and 27-OHC is not acceptable.
1200	55	30	≪ 1.5	10	Co-elution of 25-, 24S- and 27-OHC.
1200	55	34	≪ 1.5	5.5	Co-elution of 22R-, 25-, 24S- and 27-OHC.
1200	55	35	≪ 1.5	5	Co-elution of 22R-, 25-, 24S- and 27-OHC.
1200	55	36	≪ 1.5	4	Co-elution of 22R-, 25-, 24S- and 27-OHC.
1300	55	26	≈ 1.0	> 20	Analysis time too long.
1300	55	27	< 1.5	16	Co-elution of 25- and 24S-OHC.
1300	55	28	< 1.5	13	Co-elution of 25-, 24S- and 27-OHC.
1300	55	29	< 1.5	10	Co-elution of 25-, 24S- and 27-OHC.
1300	55	31	≪ 1.5	7	Co-elution of 25-, 24S- and 27-OHC.
1300	55	32	≪ 1.5	6	Co-elution of 22R-, 25-, 24S- and 27-OHC.
1300	55	34	≪ 1.5	5	Co-elution of 22R-, 25-, 24S- and 27-OHC.
1300	55	35	≪ 1.5	4	Co-elution of 22R-, 25-, 24S- and 27-OHC.
1500	55	25	≈ 1.0	> 20	Suspected leakage in LC system, analysis time too long.
1500	55	26	≈ 1.0	18	Co-elution of 25- and 24S-OHC, analysis time too long.

Table S 13: *An investigation of the in-house packed 0.1 mm ID column, continued from* ***Table S 12****.*

Flow rate µL/min	Temperature °C	Unknown gradient elution (% B)	Resolution	Analysis time (minutes)	Effect / comments
1500	55	27	< 1.5	15.5	Co-elution of 25- and 24S-OHC.
1500	55	28	< 1.5	11.5	Co-elution of 25-, 24S- and 27-OHC.
1500	55	29	< 1.5	9.5	Co-elution of 25-, 24S- and 27-OHC.
1500	55	30	< 1.5	8	Co-elution of 25-, 24S- and 27-OHC.
1500	55	31	≪ 1.5	6.5	Co-elution of 25-, 24S- and 27-OHC.
1500	55	32	≪ 1.5	5.5	Co-elution of 25-, 24S- and 27-OHC.
1500	55	33	≪ 1.5	5	Co-elution of 22R-, 25-, 24S- and 27-OHC.
1500	55	34	≪ 1.5	4.5	Co-elution of 22R-, 25-, 24S- and 27-OHC.
1500	55	35	≪ 1.5	4	Co-elution of 22R-, 25-, 24S- and 27-OHC.
1500	55	36	≪ 1.5	3	Co-elution of 22R-, 25-, 24S- and 27-OHC.

Table S 14: *An investigation of the in-house packed 0.1 mm ID column. The MP A consisted of MeOH/H_2O 5/95 (v/v) and the MP B consisted of MeOH/ACN 30/70 (v/v), both with 0.1% FA.*

Flow rate µL/min	Temperature °C	Unknown gradient elution (% B)	Resolution	Analysis time (minutes)	Effect / comments
1300	55	26	< 1.5	> 20	Co-elution of 25- and 24S-OHC, analysis time too long.
1300	55	27	< 1.5	> 20	Co-elution of 25- and 24S-OHC, analysis time too long.
1300	55	28	< 1.5	> 20	Co-elution of 25- and 24S-OHC, analysis time too long.
1300	55	29	< 1.5	18	Co-elution of 25- and 24S-OHC.
1300	55	31	≪ 1.5	12	Co-elution of 25-, 24S- and 27-OHC.
1300	55	32	≪ 1.5	10	Co-elution of 25-, 24S- and 27-OHC.
1300	55	35	≪ 1.5	6	Co-elution of 25-, 24S- and 27-OHC.
1500	55	26	< 1.5	> 20	Co-elution of 25- and 24S-OHC, analysis time too long.
1500	55	28	< 1.5	20	Co-elution of 25- and 24S-OHC.
1500	55	29	< 1.5	15.5	Co-elution of 25- and 24S-OHC.
1500	55	30	< 1.5	14	Co-elution of 25- and 24S-OHC.
1500	55	31	< 1.5	11.5	Co-elution of 25- and 24S-OHC.
1500	55	32	≪ 1.5	9	Co-elution of 25-, 24S- and 27-OHC.
1500	55	33	≪ 1.5	8	Co-elution of 25-, 24S- and 27-OHC.
1500	55	34	≪ 1.5	6.5	Co-elution of 25-, 24S- and 27-OHC.
1500	55	35	≪ 1.5	6	Co-elution of 25-, 24S- and 27-OHC.
1500	55	36	≪ 1.5	5	Co-elution of 25-, 24S- and 27-OHC.

6.7 Additional results for the method development

6.7.1 The Waters columns

Two columns from Waters was investigated due to their alternative SP and because of the previous work on separation of sterols and vitamins using these columns (sterols and vitamins with similar structures as the oxysterols) [122, 123, 138, 139]. Both these columns separate oxysterols on the basis of hydrophobic, dipole-dipole and π-π interactions (suggested by author).

Waters ACQUITY UPC^2 Torus 2-PIC (2-Picolylamine)

The Waters Torus 2-PIC column has 1.7 µm totally porous particles, illustrated in ***Figure S 7***. The column will provide (according to their homepage) excellent peak shape and selectivity towards a wide range of compounds (*e.g.* sulfamethoxazole and amitriptyline) [119].

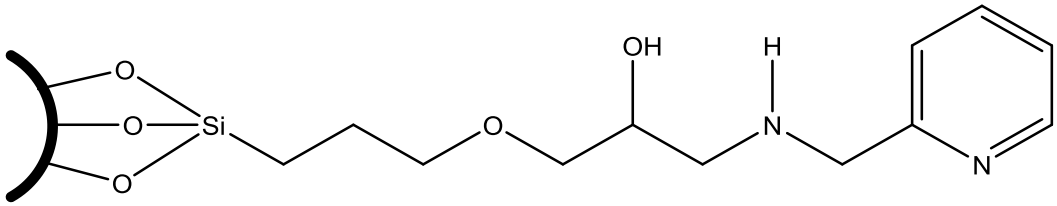


Figure S 7: *The SP with 2-picolylamine from Waters.*

Waters ACQUITY UPC^2 Torus 1-AA (1-Aminoanthracene)

The Waters Torus 1-AA column has 1.7 µm totally porous particles, illustrated in ***Figure S 8***. The column is designed for the separation of neutral compounds (polar and non-polar steroids), as well as lipids and vitamins [119].

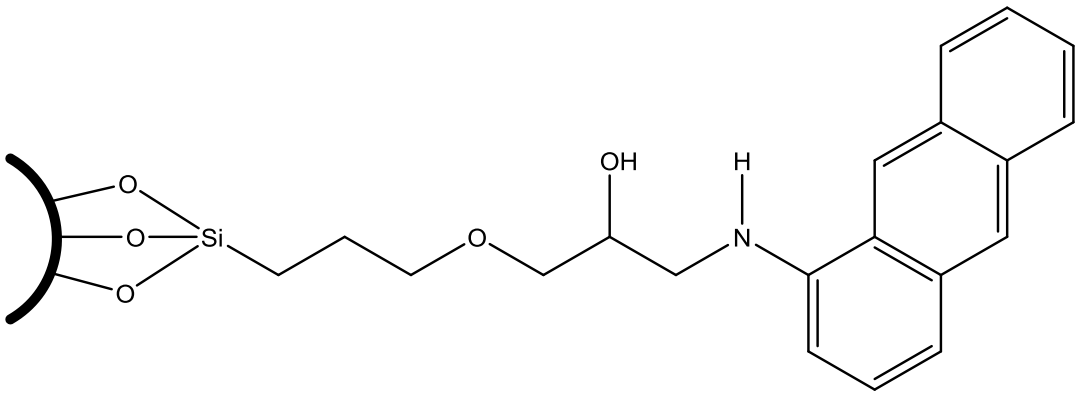


Figure S 8: *The SP with 1-aminoanthracene from Waters.*

Investigation of the Waters ACQUITY CPC2 Torus 1-AA column

The Torus 1-AA column is designed for the separation of neutral, polar and non-polar steroids, so there were high expectations for this column regarding the separation of the oxysterols, 22R-, 24S-, 25-, 27- and 22S-OHC. The initial method conditions were at room temperature (20 °C), 70% MeOH in the MP and with a 200 µL/min flow rate. These conditions made all the analytes elute in one peak. New conditions with a lower percentage of MeOH in the MP was performed to try to enhance the separation, but without success. Chromatograms are shown in ***Figure S 9***.

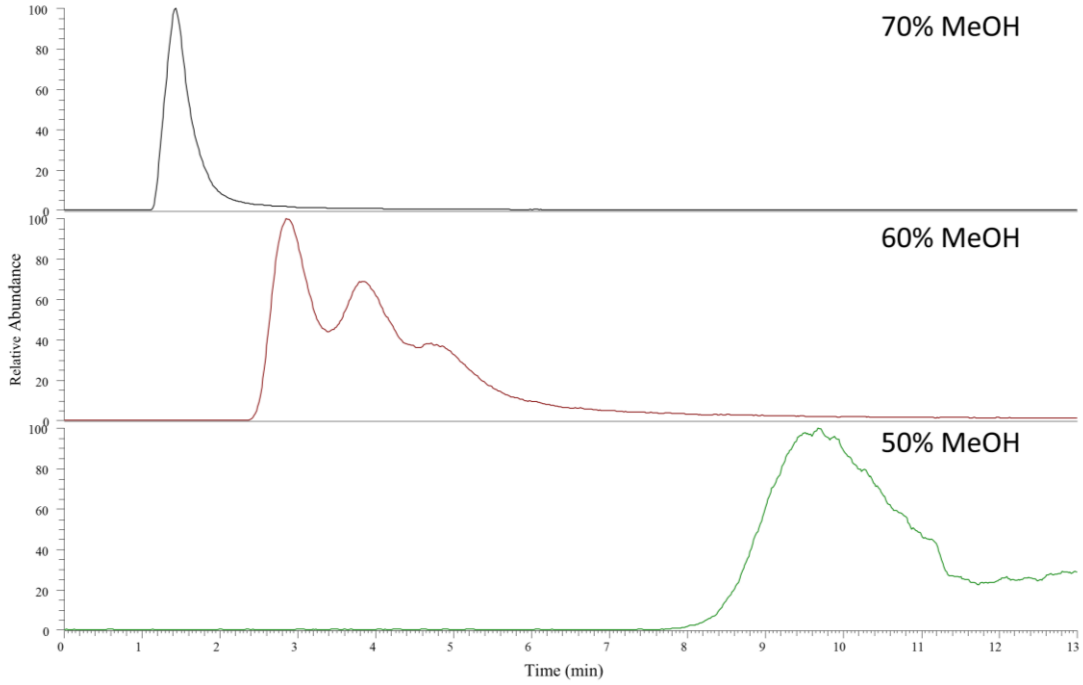


Figure S 9: *TIC chromatograms (7 points smoothing) of* m/z *514.4 → 455.4 of a standard solution containing 22R-, 24S-, 25-, 27- and 22S-OHC (2.8 µg/mL each). The separation was done with the Torus 1-AA column by using the system described in* ***Section 2.5.1****. The column temperature was 20 °C and the flow rate was 200 µL/min with different % MeOH in the MP.*

With 60% MeOH in the MP three peaks could be observed, but without enough resolution to identify the different analytes. With 50% MeOH, the analysis time was more than 15 minutes (analysis was set to end at 15 minutes). To improve the separation and get all five analytes separated, different temperatures were explored. As 60% MeOH eluted the analytes too fast and 50% gave to low retention of the analytes, the amount of organic solvent was set to 55% with a flow rate of 150 µL/min. This gave the chromatograms shown in ***Figure S 10***. The change in temperature in the column oven did not give the desired outcome. Instead of separating the analytes, the peaks became broader without base line separation. With 60 °C in

the column oven and 55% MeOH in the MP the outcome was similar to the analysis with 20 °C and 70% MeOH in the MP (***Figure S 9***).

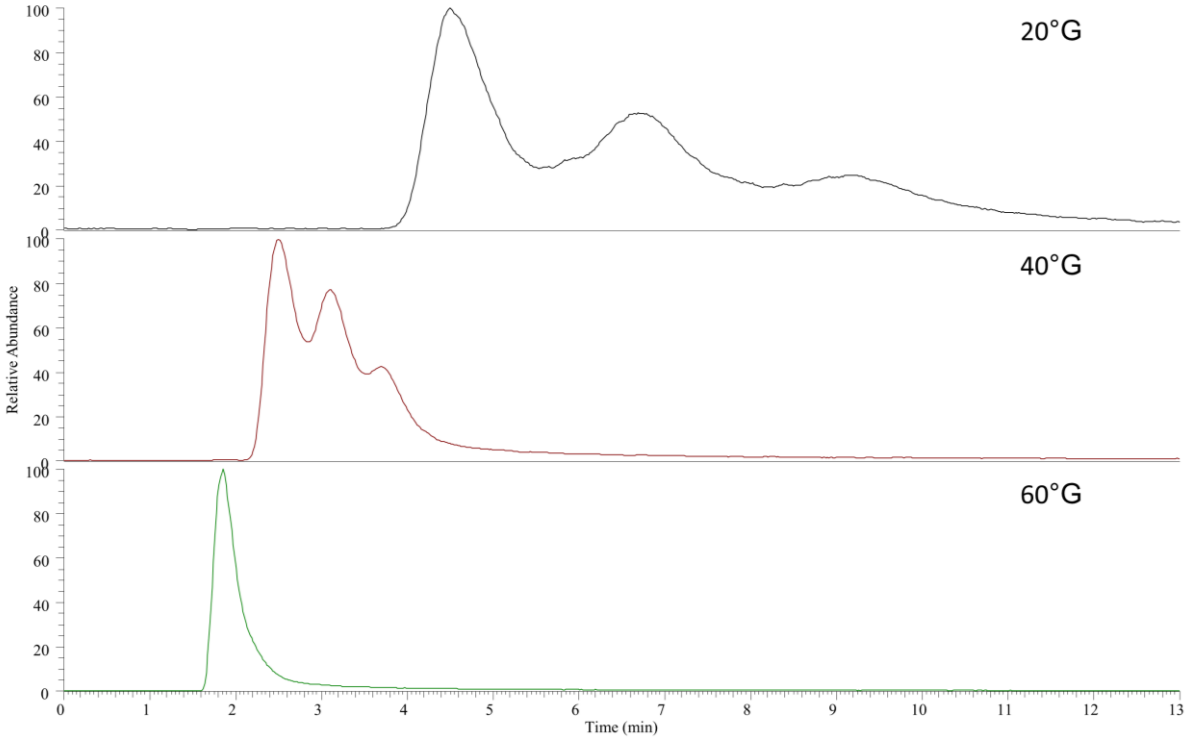


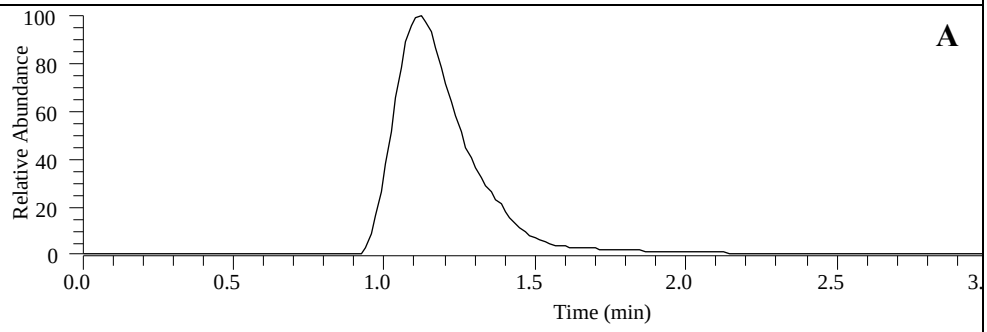
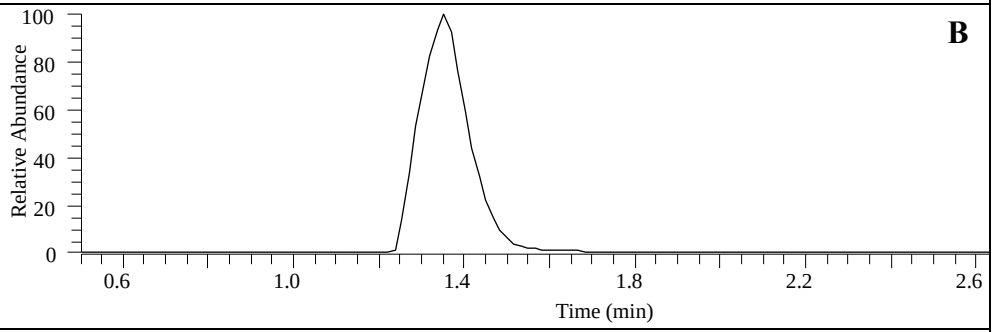
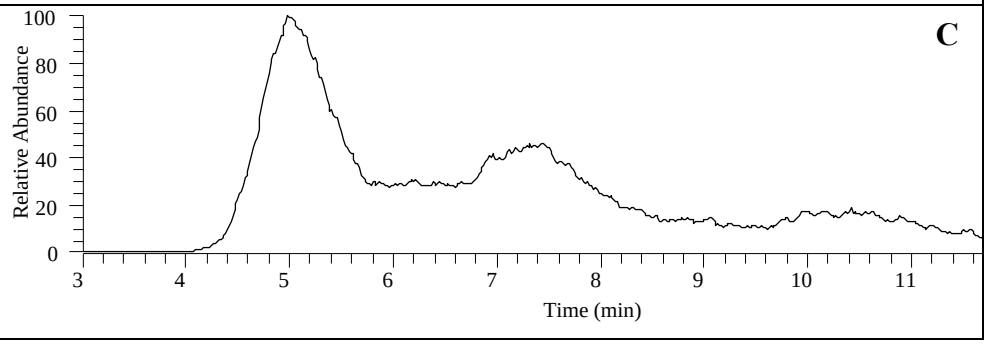
Figure S 10: *TIC chromatograms (7 points smoothing) of* m/z *514.4 → 455.4 of a standard solution containing 22R-, 24S-, 25-, 27- and 22S-OHC (2.8 µg/mL each). The separation was done with the Torus 1-AA column by using the system described in* ***Section 2.5.1****. The MP consisted of 55% MeOH with a flow rate of 150µL/min at different temperatures.*

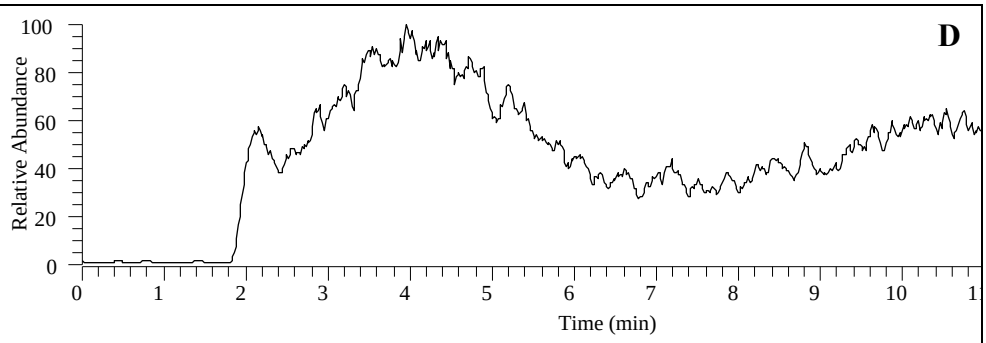
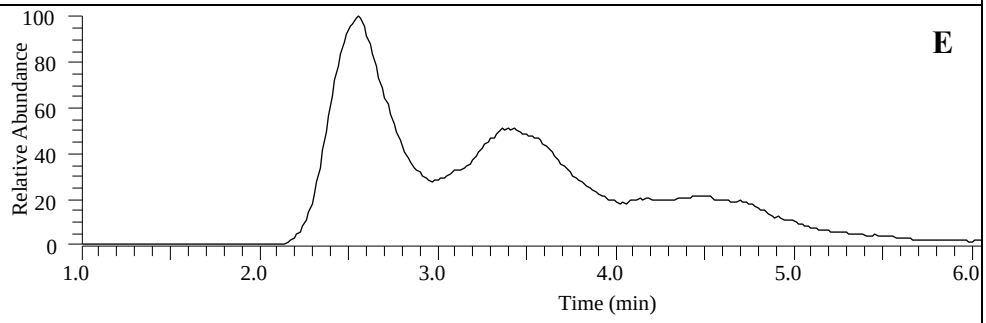
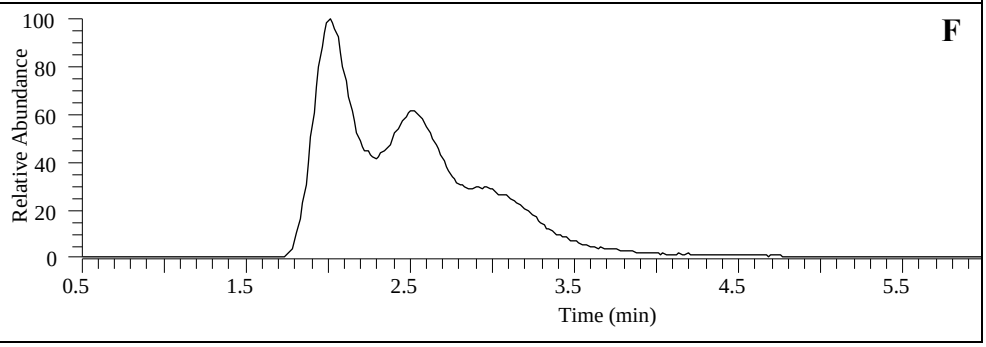
Analyses with 45% MeOH in the MP and 60 °C and 40 °C in the column oven gave similar chromatograms as shown in ***Figure S 10*** for 20 °C and 40 °C with 55% MeOH in the MP.

To attempt to get alternative selectivity for the analytes and perhaps improved separation, a different organic modifier in the MP was tested. EtOH is less polar than MeOH and therefore a stronger eluent for hydrophobic analytes such as oxysterols [140]. The consequence of this effect was proven after analyzing the mixture of oxysterols with 70% EtOH as the MP B with a flow rate of 200 µL/min and column temperature at 30 °C. All the analytes eluted at t_M in one peak. The outcome was the same when using 50% EtOH and 40% EtOH. At 30% EtOH in the MP, the result was similar to the one with 55% MeOH, 20 °C in the column oven and 150µL/min flow (upper chromatogram in ***Figure S 10***).

As an alternative attempt to separate the analytes, a mixture of MeOH as the MP B and EtOH as the MP C was examined, and which is a common trick to get different selectivity [141, 142]. By mixing MeOH and EtOH as the MP B and C, the properties from each organic modifier could impact the separation in a different way [143]. The different mixtures ratios of EtOH and MeOH used are shown in ***Table S 15***.

Table S 15: *Chromatograms with different amounts of MeOH (pump channel B) and EtOH (pump channel C) (both with 0.1% FA) as the MP (pump A delivers H_2O with 0.1% FA and provides the remaining MP so that total amount is 100%). The chromatograms in the table are presented as TIC chromatograms of* m/z *514.4 → 455.4 of a standard solution containing 22R-, 24S-, 25-, 27- and 22S-OHC (1.0 µg/mL each). Separation was done with the Torus 1-AA column by using the system described in* ***Section* 2.5.1**. *The temperature was 20 °C and the flow rate was 200 µL/min.*

v/v		Chromatogram
MeOH (B)	EtOH (C)	
30%	30%	
25%	25%	
22%	22%	

20%	20%	
30%	20%	
20%	30%	

As shown in ***Table S 15***, a MP combination of MeOH, EtOH and H_2O did not improve the separation. Six different compositions of MeOH and EtOH were tested, where **A** and **B** in ***Table S 15*** resulted in co-elution of the analytes in one peak. The compositions and associated chromatograms **C**, **E** and **F** shows indication of three peaks, but R_S was too low ($R_S \ll 1$) to clearly identify the analytes. With 20% MeOH and 20% EtOH, as shown in chromatogram **D**, the peaks eluted in ≈5 min, and which indicates that this MP composition was not able to elute the analytes from the column in a reasonable time.

As the column failed at separating the mixture of 22R-, 24S-, 25-, 27- and 22S-OHC it was concluded not to proceed further with this column.

Investigation of the Waters ACQUITY CPC2 Torus 2- PIC column

The Torus 2-PIC (illustrated in ***Figure S 7***) has a SP where the functional group is similar to the sterol ring structure of the analytes. A rule of thumb in chromatography states that analytes have god affinity towards SP's that are similar to themselves, and thereby has a better possibility to get separation. The suggested interaction between the analytes and the SP in this column will mainly be towards the ring structure [144].

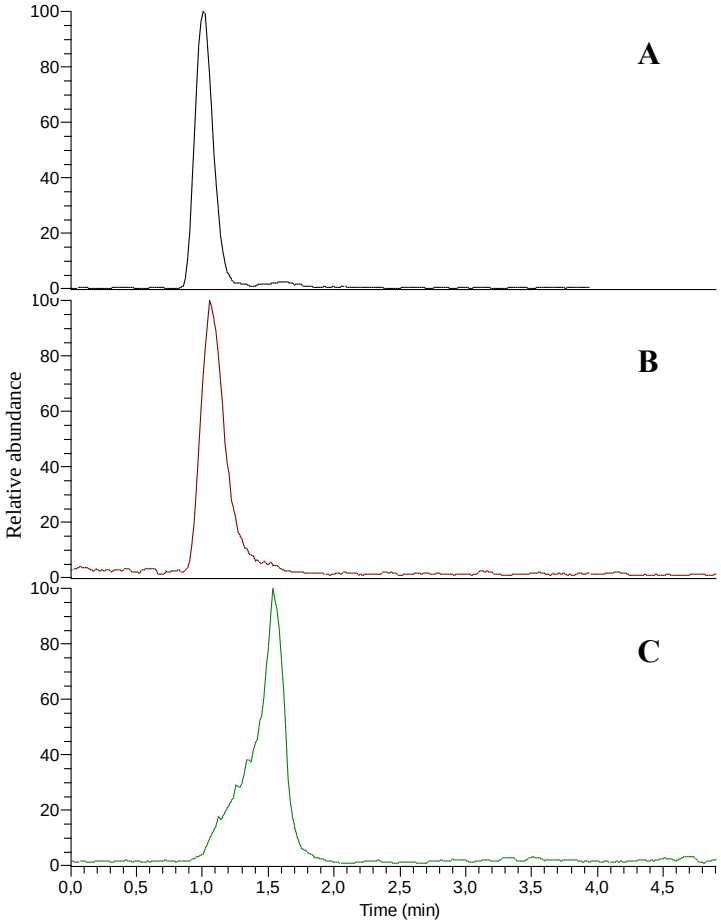


Figure S 11: *TIC chromatograms (7 points smoothing) of m/z 514.4 → 455.4 of a standard solution containing 22R-, 24S-, 25-, 27- and 22S-OHC (2.8 µg/mL each). Separation was done with the Torus 2-PIC column by using the system described in* ***Section 2.5.1****. The flow rate was 170 µL/min at 25 °C where* ***A****) The MP consisting of* H_2O*/MeOH 25/75 (v/v),* ***B****) The MP consisting of* H_2O*/MeOH 50/50 (v/v) and* ***C****) the MP consisting of* H_2O*/MeOH 70/30 (v/v), all with 0.1% FA*

As preliminary condition for the analysis of a mixture of 22R-, 24S-, 25-, 27-, and 22S-OHC, the flow rate was set to 200 µL/min with 75% MeOH as the MP B at 25 °C. With these conditions, all the analytes eluted as one peak, as shown in **A** in ***Figure S 11.*** When the % MeOH in the MP was adjusted from 75% to 50%, the outcome was the same as when using 75% MeOH (**B** in ***Figure S 11*****)**. As a last attempt to get retention of the analytes, 30% MeOH in the MP was used. This gave one broad peak with fronting as shown in chromatogram **B** in ***Figure S 11***.

30% organic solvent in the MP with a more hydrophobic SP would not be enough organic solvent to even elute the analytes, as observed for *e.g.* ACE SPH and ACE SuperC18 column. To examine the retention times of the analytes, each analyte was analyzed separately with

condition as shown in chromatogram **A** in ***Figure S 11.*** This provided the chromatogram as shown in ***Figure S 12***. All the analytes eluted at the same retention time, with no affinity towards the SP.

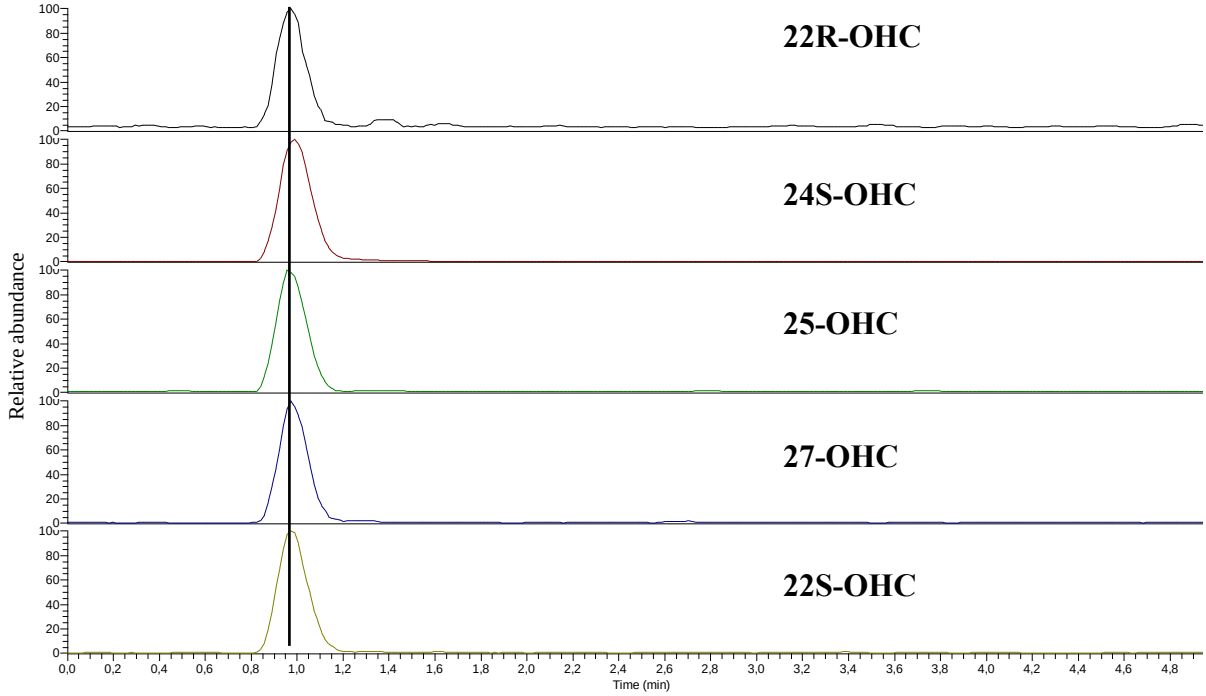


Figure S 12: *TIC chromatograms (7 points smoothing) of* m/z *514.4 → 455.4 of each analyte (2.8 µg/mL). The separation was done with the Torus 2-PIC column by using the system described in* ***Section 2.5.1****. The flow rate was 170 µL/min at 25 °C with the MP consisting of* H_2O*/MeOH 25/75* (v/v) *with 0.1% FA.*

To make sure that it is possible to get retention with this SP, two other compounds were chromatographed on the Torus 2-PIC column. Sulfamethoxazole (***Figure S 13***) and propranolol (***Figure S 14***) were used to control the retention on the SP.

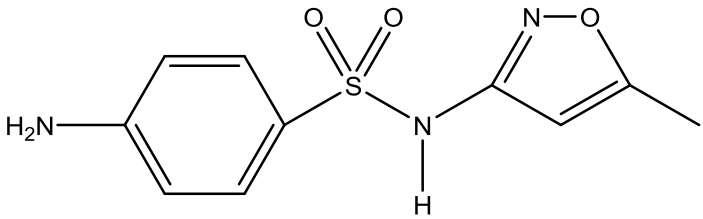


Figure S 13: *The chemical structure of sulfamethoxazole.*

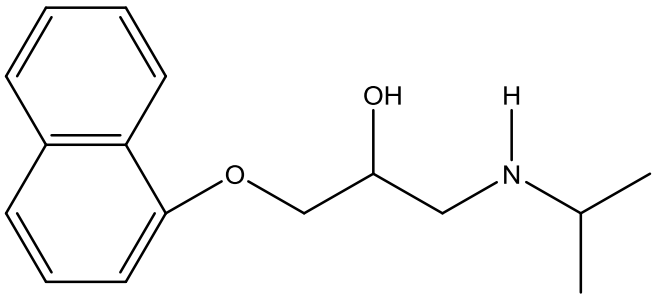


Figure S 14: *The chemical structure of propranolol.*

Both Sulfamethoxazole and propranolol was analyzed in negative mode with two different MP compositions; 65% MeOH and 75% MeOH. As shown in ***Figure S 15***, the two compounds were easily separated. This confirmed that the SP provides retention of the two antibiotics. Hence, with chromatogram **A** shown in ***Figure S 11***, this indicates that the SP does not have enough selectivity towards the oxysterols and are therefore not able to separate them.

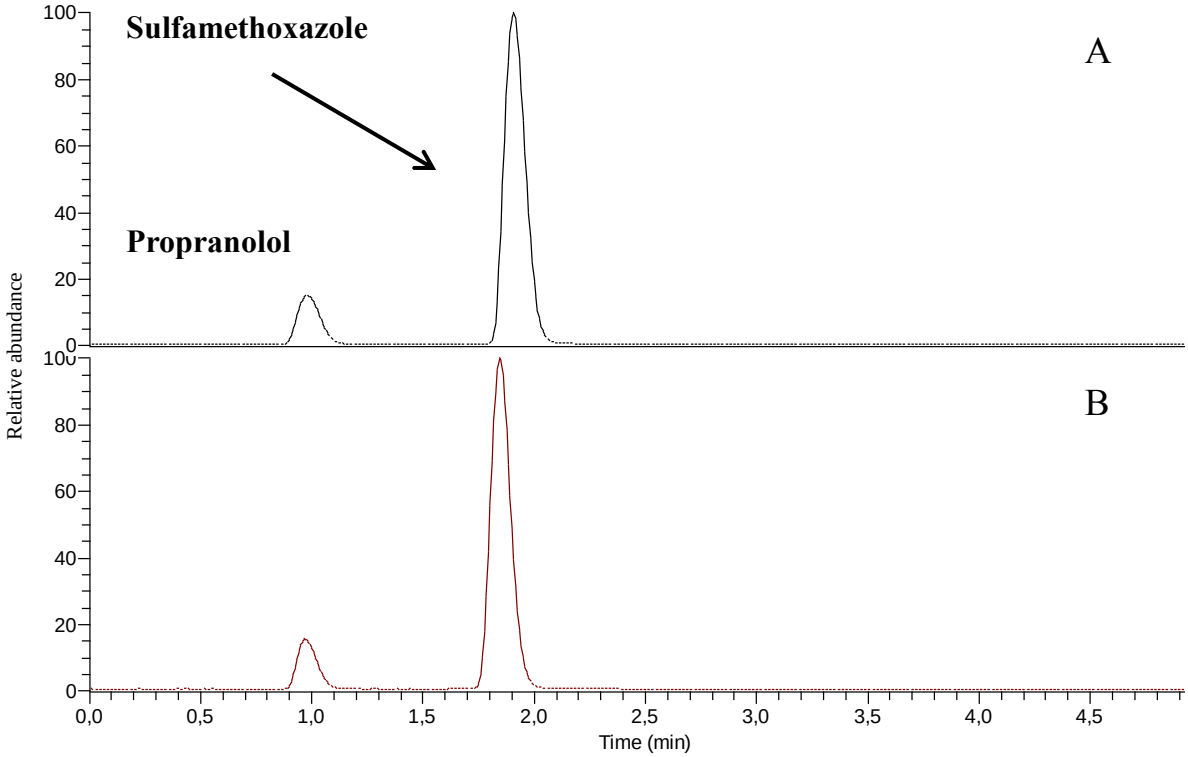


Figure S 15: *TIC chromatograms of m/z 259.6 → 157.0 (propranolol) and m/z 253.6 → 156.0 (sulfamethoxazole) in a mixture of sulfamethoxazole and propranolol (6.6 µg/mL each). The separation was done with the Torus 2-PIC column by using the system described in* ***Section 2.5.1****. The flow rate was 170 µL/min at 25 °C where* ***A)*** *The MP consisting of H_2O/MeOH 35/65 (v/v) and* ***B)*** *The MP consisting of H_2O/MeOH 25/75 (v/v), both with 0.1% FA.*

6.7.2 Investigation of the Thermo Scientific Hypersil Gold C_{18} column

The Hypersil Gold C_{18} column has a silica totally porous structure with C_{18} as the functional group attached to it. The Hypersil Gold C18 column has been applied several times for the separation of oxysterols [52, 103, 145].

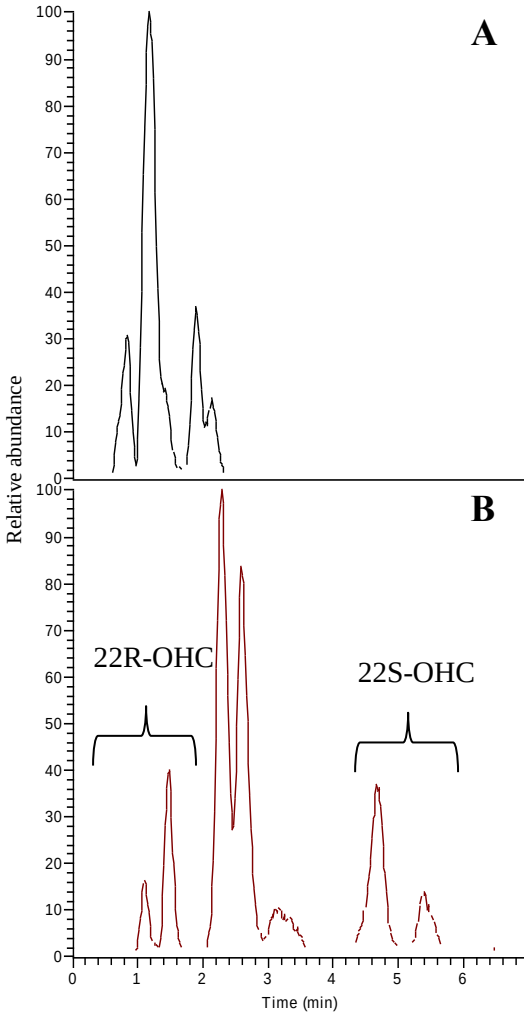


Figure S 16: *TIC chromatograms (7 points smoothing) of m/z 514.4 → 455.4 of a standard solution containing 22R-, 24S-, 25-, 27- and 22S-OHC (2.8 µg/mL each). Separation was done with the Thermo Hypersil Gold C18 column by using the system described in* ***Section 2.5.1****. The flow rate was 170 µL/min at 25 °C where* ***A****) The MP consisting of H_2O/MeOH 25/75 (v/v) and* ***B****) The MP consisting of H_2O/MeOH 30/70 (v/v), both with 0.1% FA.*

As with the ACE columns, this column was also tested systematical (not shown). The systematical testing did not provide any information of what MP, temperature or flow rate that was optimal, so a step-by-step approach was initiated. The investigation of the Hypersil Gold column continued where analysis was performed with a high amount of organic solvent in the MP (75% MeOH), column temperature at 25 °C and a flow rate of 170 µL/min. With these settings, there were three separated peaks in the chromatogram and which indicates that 24S-, 25-, and 27-OHC are co-eluting (chromatogram **A** in ***Figure S 16***). The amount of MeOH in the MP was adjusted to 70%, with the same temperature and flow rate. This gave better separation, but not sufficient ($R_S \ll 1.5$) regarding resolution of 24S-, 25- and 27-OHC as seen in chromatogram **B** in ***Figure S 16***. The retention order for the analytes in the latter chromatogram that was eluting between 2 and 4 minutes was 24S-OHC, 25-OHC and 27-OHC.

When adjusting the amount of organic solvent in the MP to 65% MeOH, the separation of 24S-, 25-

and 27-OHC was slightly improved (chromatogram not shown), but at the expense of s longer analysis time (over 15 min).

The retention times for the analytes decreases when the temperature is increased. Also, the backpressure is reduced at higher temperatures, meaning a higher flow rate can be used, hence more rapid analysis is attainable (***Equation 7*** *and* ***Equation 8***). As an example, 25 °C versus 60 °C in the column oven made it possible to increase the flow rate from 170µL/min to 360µL/min without any significant increase in pressure. The disadvantage of increasing the temperature is that a lower amount of organic solvent in the MP can be used (to avoid analytes eluting too fast), and that could give complications regarding solubility of the oxysterols (not soluble in too much water) [146]. Higher temperature did reduce the analysis time but provided poor resolution between the analytes, as shown in ***Figure S 17***.

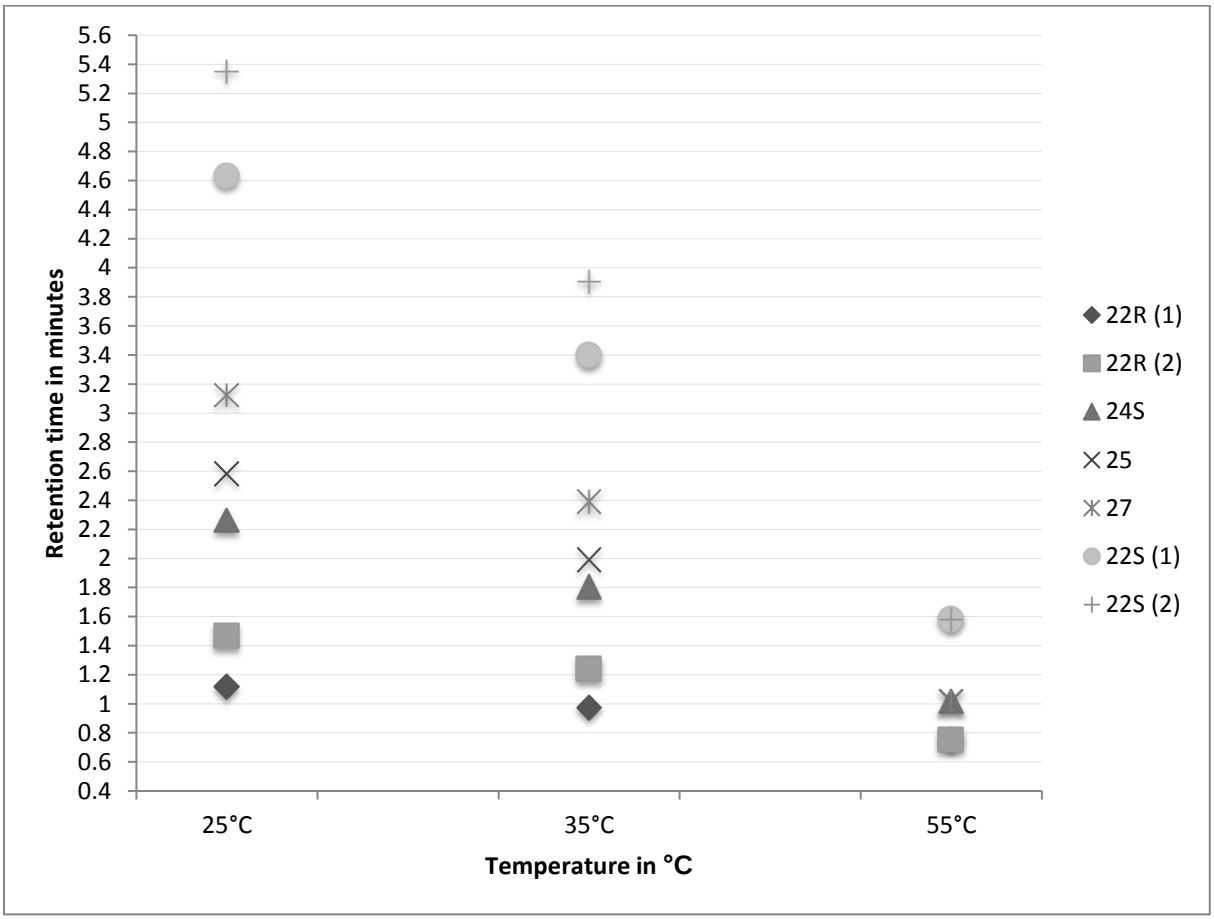


Figure S 17: *The retention times at three different temperatures; 25 °C, 35 °C and 55 °C. The separation was done with the Thermo Hypersil Gold C18 column and by using the system described in* ***Section 2.5.1****. The flow rate was 170 µL/min with a MP composition of H_2O/MeOH 30/70 (v/v) with 0.1% FA.*

Investigation of different MP compositions at high temperature and high flow rate was also examined. High temperature can improve the peak shape, hence improve the resolution [58]. As seen in ***Figure S 18***, high temperature and flow rate with different MP compositions did not give the desired outcome with the desired resolution ($R_S \approx 1.5$).

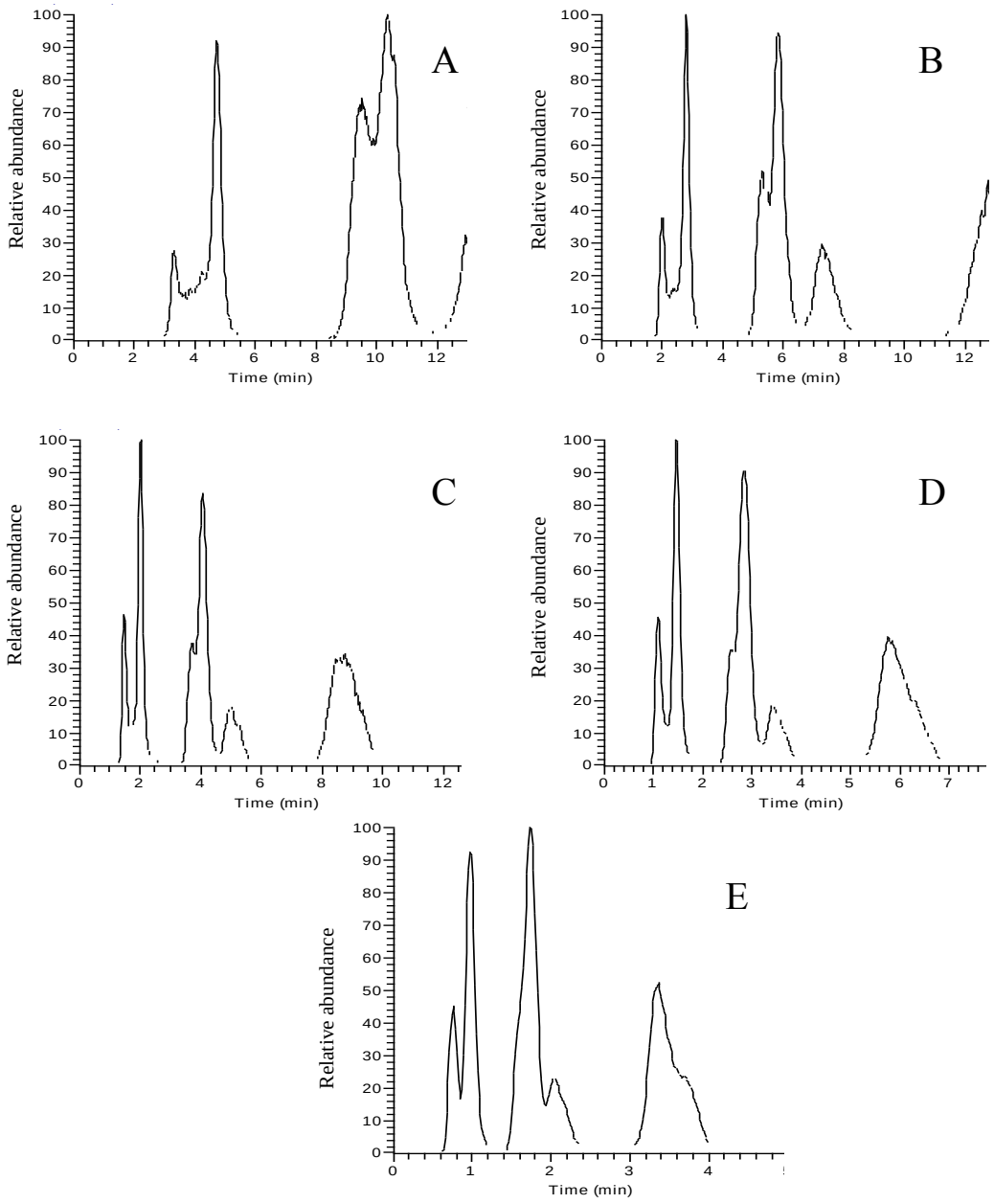


Figure S 18: *TIC chromatograms (7 points smoothing) of m/z 514.4 → 455.4 of a standard solution containing 22R-, 24S-, 25-, 27- and 22S-OHC (2.8 µg/mL each). The separation was done with the Thermo Hypersil Gold C18 column by using the system described in* ***Section 2.5.1****. The flow rate was 360 µL/min at 60 °C where the MP consisted of* ***A****) H_2O/MeOH 50/50 (v/v),* ***B****) H_2O/MeOH 47/53 (v/v),* ***C)*** *H_2O/MeOH 45/55 (v/v),* ***D)*** *H_2O/MeOH 43/57 (v/v) and* ***E)*** *H_2O/MeOH 40/60 (v/v), all with 0.1% FA.*

As an alternative attempt to improve the resolution, a reduced temperature was examined. As the viscosity increases at lower temperature, a reduced flow rate had to be utilized to avoid overloading the pump considering the increased pressure (***Equation 7)***. Hence, a flow rate of 100 µL/min with a MP consisting of H_2O/MeOH 70/30 (*v/v*) at a temperature of 20 °C was examined as shown in ***Figure S 19***.

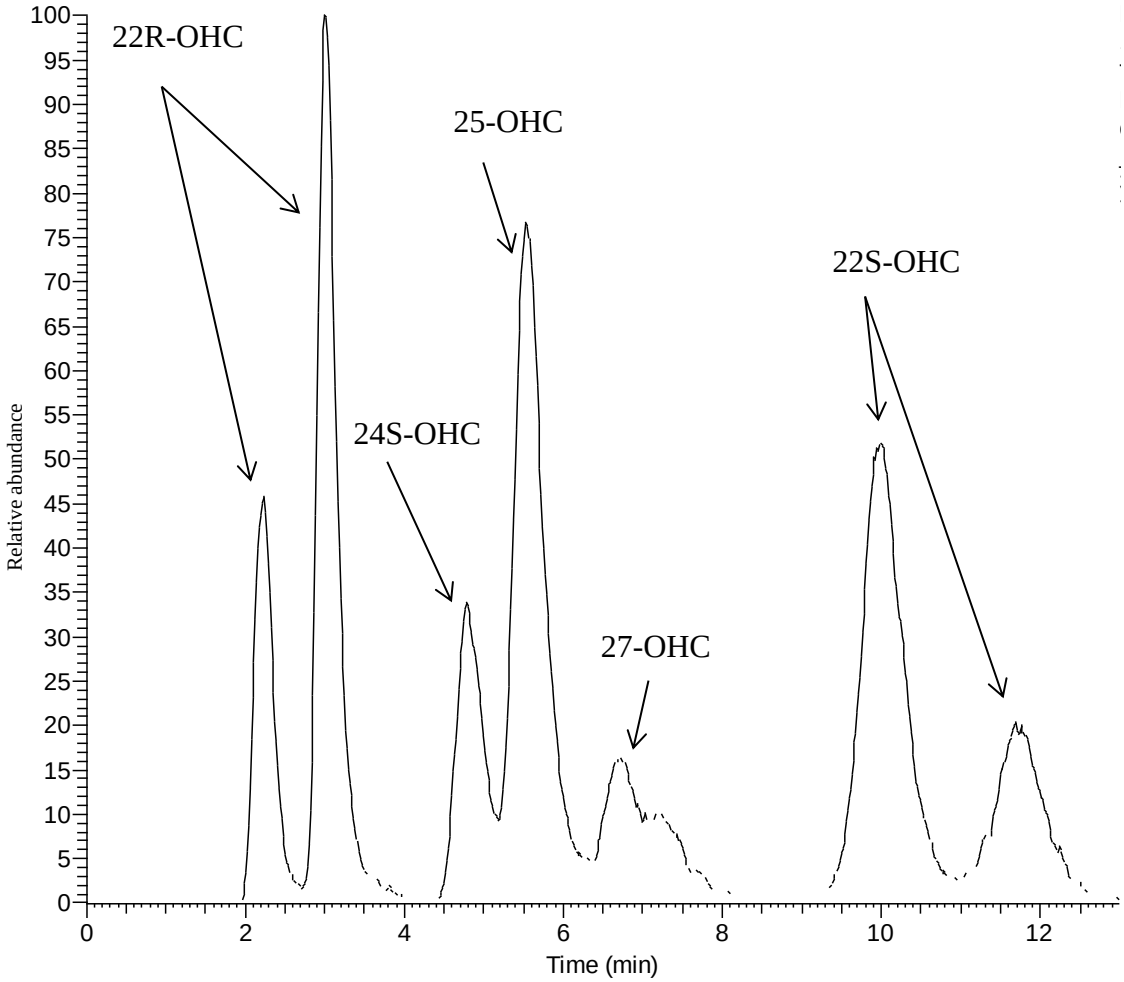


Figure S 19: *A TIC chromatogram (7 points smoothing) of m/z 514.4 → 455.4 of a standard solution containing 22R-, 24S-, 25-, 27- and 22S-OHC (2.8 µg/mL each). The separation was done with the Thermo Hypersil Gold C18 column by using the system described in* ***Section 2.5.1****. The flow rate was 100 µL/min at 20 °C where the MP consisted of H_2O/MeOH 30/70 (v/v) with 0.1% FA.*

The resolution between 24S-OHC and 25-OHC in ***Figure S 19*** was 1.2. The pressure was ≈300 bars with the conditions in ***Figure S 19***, so a minor increase of flow rate together with a decrease in temperature to 15 °C was examined. This provided the best chromatographic performance considering resolution, but at the cost of the analysis time as shown in ***Figure 27***.

The resolution and peak symmetry got poorer at temperature below 15 °C and at higher flow rates (chromatograms not shown). Different MP compositions was explored to reduce the analysis time, but without success. Different organic solvents (ACN and EtOH) did not provide different selectivity either, so the conditions used to obtain the chromatogram in ***Figure 27*** turned out to be the best choice for this column.

With these conditions (flow rate, temperature and MP composition), the column provided better chromatographic performance compared to the ACE Super C18 column. The resolution between 24S-OHC and 25-OHC was 1.4 with the Hypersil Gold C18 column, but the analysis time was too long. As seen in ***Figure 27***, only one of the two peaks that represents 22S-OHC eluted within the method analysis time (was set to end at 13 minutes).

As core-shell particles provided the ability to reduce the analysis time and still maintain a high efficiency (hence good resolution), no more experiments were conducted with the Hypersil Gold C18 column. Even so, the investigation of this column showed the most promising performance in the beginning of this study, and with the conditions used to obtain the chromatogram in ***Figure 27***, an improvement regarding the analysis time was achieved compared to the results obtained by Honda *et al.* [52]. They used a Hypersil Gold C18 column (150 x 2.1 mm ID with 3 µm particles) and was able to separate 24S-, 25- and 27-OHC in 17 minutes. With the conditions used to obtain the chromatogram in ***Figure 27***, the separation of the same oxysterols was performed in 13 minutes, and which correspond to a 30% improvement.

6.7.3 The 2.1 mm ID ACE SuperPhenylHexyl column – General effects of the amount of methanol in the mobile phase, the temperature and the flow rate

The ACE SPH column was examined to map the general effects of the amount of MeOH, the temperature and the flow rate. 24S-OHC was not included in the mixture during the examination of general effects due to co-elution with 25-OHC.

Effect of MeOH as organic modifier

The amount of MeOH in the MP was examined with a flow rate of 250 µL/min at 25 °C and the outcome of this is shown in ***Figure S 20***. A standard solution containing 22R-, 25-, 27-, and 22S-OHC (2.8 µg/mL each) was prepared with the sample preparation method described in ***Section 2.2.2***.

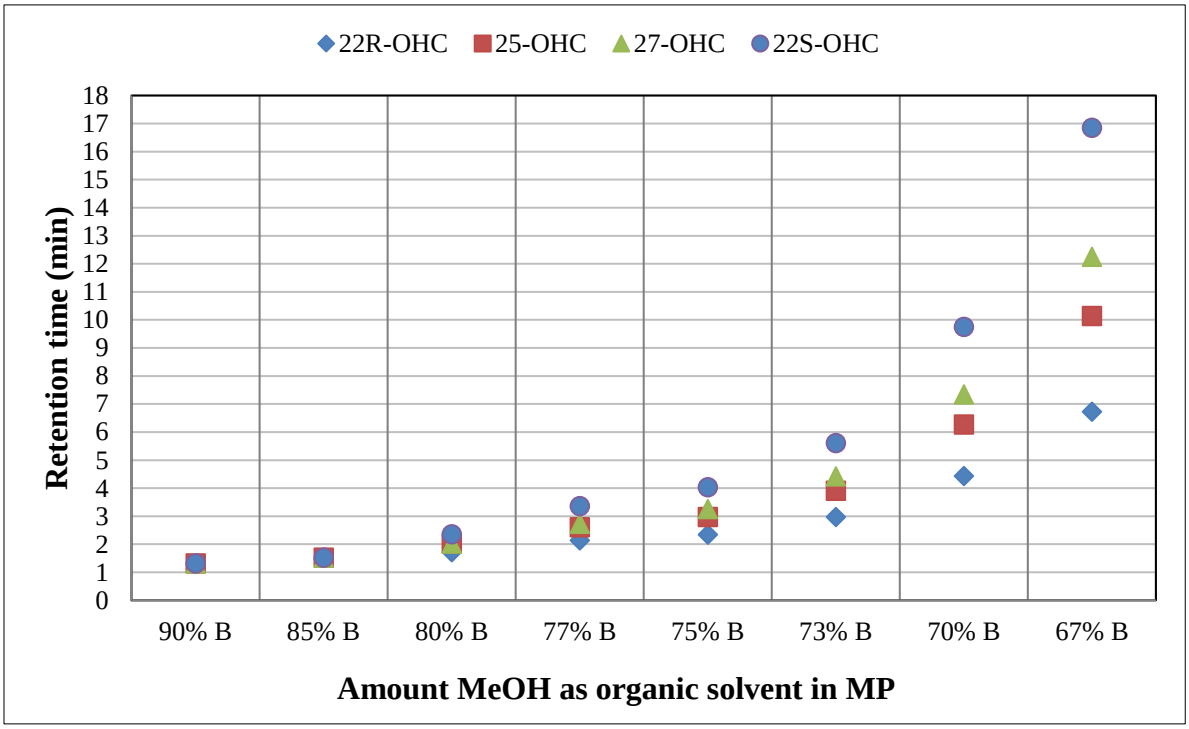


Figure S 20: *The retention times with different amounts of MeOH as the organic solvent in the MP composition was examined. The data were obtained by analyzing a standard solution containing 22R-, 25-, 27- and 22S-OHC (2.8 µg/mL each) with the 2.1 mm ID ACE SPH column and the system described in* ***Section 2.5.1.*** *The flow rate was 250 µL/min at 25 °C.*

The amount of MeOH in the MP had to be 70% or less to get separation between 25-OHC and 27-OHC. When the amount of MeOH in the MP was between 75% - 90%, co-elution prevented a secure identification of the two analytes (25-OHC and 27-OHC).

Effect of temperature

The retention time will decrease at elevated temperatures due to the decrease in viscosity and thereby improvement of the mass transfer of the analytes according to ***Equation 8***. In addition, an increased temperature allows higher flow rate as the backpressure is reduced (***Equation 7***).

When the temperature was increased, the retention time for each analyte was reduced (as predicted), ***Figure S 21***. The increased temperature affected 22S-OHC the most, where the retention time got reduced by 1.63 minutes when the temperature was adjusted from 60 °C to 70 °C (at the same flow rate and MP composition).

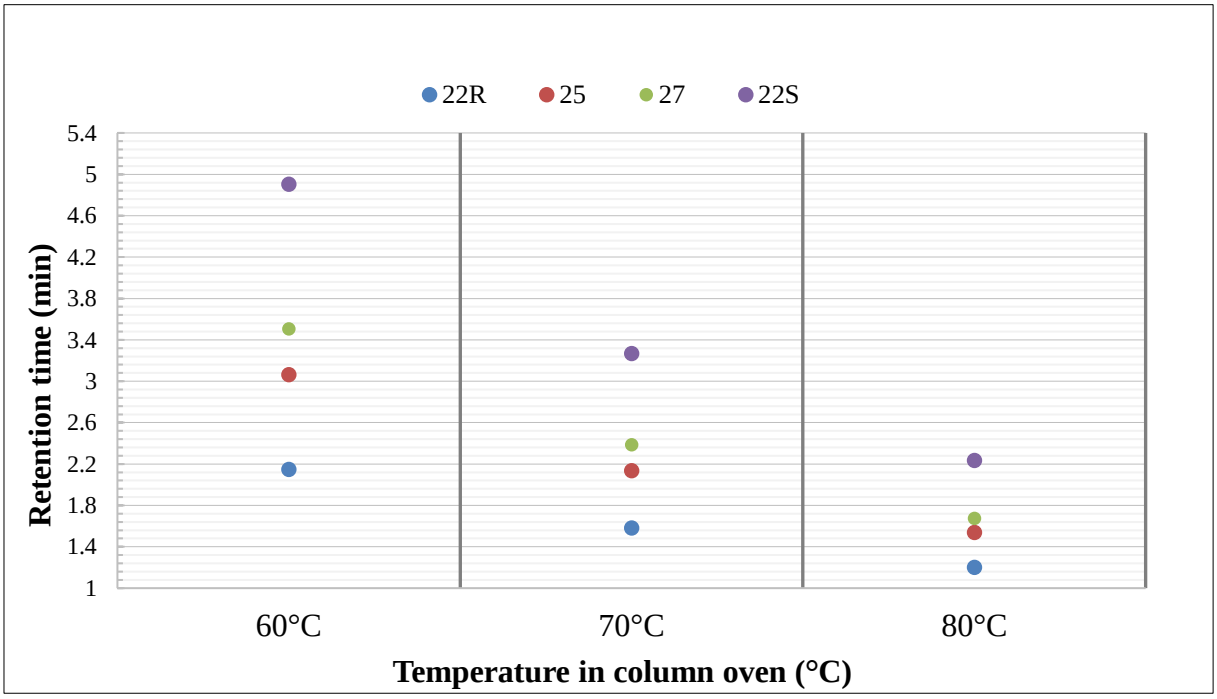


Figure S 21: *The retention times with elevated temperatures were examined. The data were obtained by analyzing a standard solution containing 22R-, 25-, 27- and 22S-OHC (2.8 µg/mL each) with the 2.1 mm ID ACE SPH column and the system described in* ***Section 2.5.1.*** *The MP composition was H_2O/MeOH 40/60 (v/v) with 0.1% FA and 500 µL/min flow rate.*

Effect of flow rate

The effect of adjusting the flow rate is shown in ***Figure S 22***. The ACE SPH column showed a similar trend as the ACE Super C18 column regarding flow rate, but the ACE SPH column was able to provide better resolution between the oxysterols at higher flow rates.

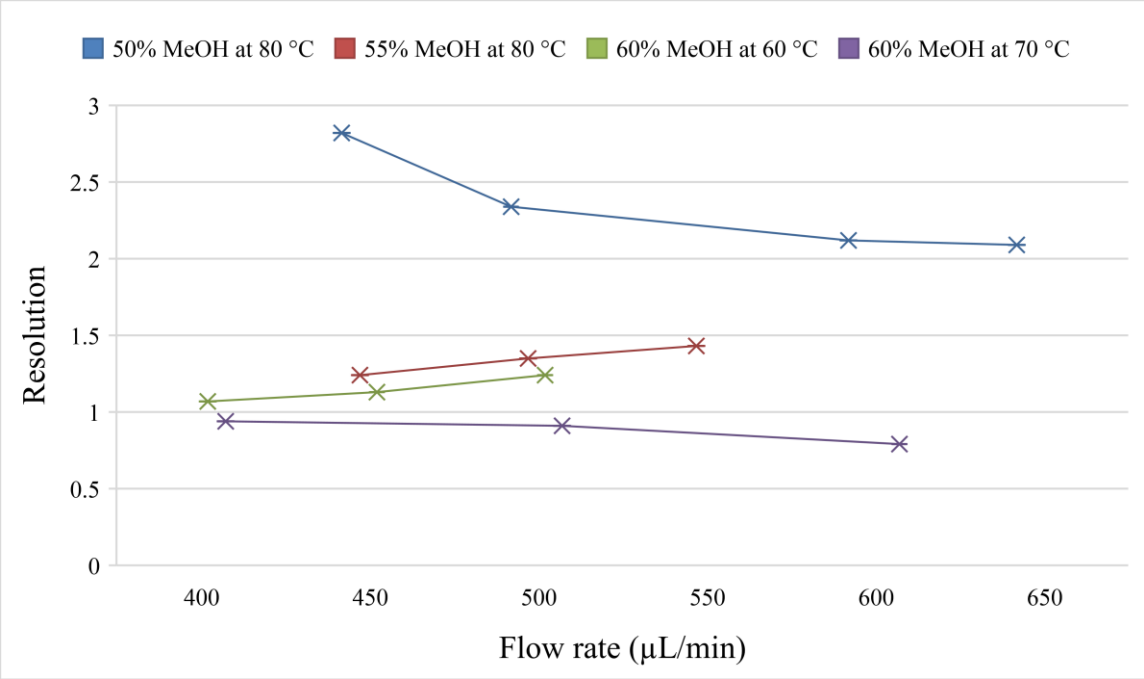


Figure S 22: *The resolution between the two closest eluting peaks; 25- and 27-OHC where different flow rates were examined. The data were obtained by analyzing a standard solution containing 22R-, 25-, 27- and 22S-OHC (2.8 µg/mL each) with the 2.1 mm ID ACE SPH column and the system described in* ***Section 2.5.1.*** *The MP composition and temperature is given in the plot.*

A high flow rate is essential to achieve a rapid analysis. Since the operating pressure is increased at high flow rates, a high temperature is also needed. The determination of oxysterols using high temperature, high flow rate and low amount of MeOH in the MP was investigated. As seen in ***Figure S 22***, the conditions that provided the best resolution was at a temperature of 80 °C (maximum temperature for the oven) with a flow rate of 450 µL/min where the MP consisted of H_2O/MeOH 50/50 (*v/v*) with 0.1% FA. However, these conditions gave an analysis time of > 10 minutes.

With a MP consisting of H_2O/MeOH 45/55 (*v/v*) with 0.1% FA, a flow rate of 550 µL/min at 80 °C, the two closely eluting compounds, 25- and 27-OHC, had an acceptable resolution of 1.4 and an analysis time of ≈ 8 minutes. However, when 24S-OHC was included in the sample mixture, it was still co-eluting with 25-OHC with the conditions mentioned above.