

High sensitivity measurements of side-chain oxysterols using liquid chromatography mass spectrometry

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Preface

The work presented in this thesis has been carried out at Department of Chemistry, University of Oslo in the time period 2012-2015 under supervision of Prof. Elsa Lundanes and Ass. Prof Steven R Wilson. Biological experiments have been performed at Unit for Cell Signaling, Rikshospitalet, Oslo University Hospital under the supervision of Prof. Stefan Krauss.

First, I would like to thank my supervisors for giving me guidance and support through this work. It has been challenging and sometimes frustrating, but most of all fun. I would also like to acknowledge all my co-authors, which have contributed to the work presented.

I would like to thank all the students and colleagues in the bioanalytical group, especially Marita Clausen for her contribution to a good working environment and Inge Mikalsen for all technical support and training. Also, I would like to thank Hege Lynne for guiding me through my apprentice time many years ago and being a support after that. A special thanks to my master students Caroline Vesterdal and Kristina E Sæterdal and bachelor student Stian Solheim for good collaboration and research. I would also like to thank all the students and colleagues in Stefan Krauss group for their contribution to this work.

Thanks to all my friends for always being there, special Sandra, Aase Marit, and Monica for fun, but also professional late night discussion. I would also like to thank Vibeke and Shari for lunches and for reminding me that chemistry is not everything in life (only almost). In the end I would like to thank my family, especially my mom and dad for always supporting me and believing in me.

Finally, Kim, this thesis is dedicated to you. Thanks for all your love and support during this work. I love you.

Hanne Røberg-Larsen,
Oslo, December 2015

List of publications

- I. Hanne Roberg-Larsen, Martin Frank Strand, Anders Grimsmo, Petter Angell Olsen, Jennifer L Dembinski, Frode Rise, Elsa Lundanes, Tyge Greibrokk, Stefan Krauss and Steven Ray Wilson. **High Sensitive measurements of active oxysterols with automated filtration/filter backflush-solid phase extraction- liquid chromatography-mass spectrometry.** Journal of Chromatography A 1255 (2012) 291-297
- II. Hanne Roberg-Larsen, Kaja Lund, Tore Vehus, Nina Solberg, Caroline Vesterdal, Dorna Misaghian, Petter Angell Olsen, Stefan Krauss, Steven Ray Wilson and Elsa Lundanes. **Highly automated nano-LC/MS-based approach for thousand cell-scale quantification of side chain-hydroxylated oxysterols.** Journal of Lipid Research 55 (2014) 1531-1536
- III. Hanne Roberg-Larsen, Caroline Vesterdal, Steven Ray Wilson and Elsa Lundanes. **Underivatized oxysterols and nanoLC-ESI-MS: A mismatch.** Steroids 99 (2015) 125-130
- IV. Hanne Roberg-Larsen, Kaja Lund, Kristina E Saeterdal, Stian Solheim, Tore Vehus, Nina Solberg, Stefan Krauss, Elsa Lundanes and Steven Ray Wilson. **Mass spectrometric detection of 27-hydroxycholesterol in breast cancer exosomes.** Journal of Steroid Biochemistry and Molecular Biology. Submitted

Additional publications related to this thesis

- SI. Kristin Opsal Svendsen, Hanne Roberg-Larsen, Siri Altern Pedersen, Ida Brenna, Elsa Lundanes and Steven Ray Wilson. **Automatic filtration and filter flush for robust online solid-phase extraction liquid chromatography.** Journal of Separation Science 34 (2011) 3020-3022
- SII. Hanne Roberg-Larsen, Martin Frank Strand, Stefan Krauss and Steven Ray Wilson. **Metabolites in vertebrate Hedgehog signaling.** Biochemical and Biophysical Research communications 446 (2014) 669-674
- SIII. Magnus Rogeberg, Helle Malerod, Hanne Roberg-Larsen, Cecilie Aas and Steven Ray Wilson. **On-line solid phase extraction-liquid chromatography, with emphasis on modern bioanalysis and miniaturized systems.** Journal of Pharmaceutical and Biomedical Analysis 87 (2014) 120-129
- SIV. Nikiana Simigdala, Qiong Gao, Sunil Pancholi, Hanne Roberg-Larsen, Marketa Zvelebil, Ricardo Ribas, Elizabeth Folkerd, Andrew Thompson, Amandeep Bhamra, Mitch Dowsett and Lesley-Ann Martin. **Cholesterol biosynthesis pathway as a novel mechanism of resistance to estrogen deprivation in estrogen receptor positive breast cancer.** Breast Cancer Research, Submitted

Abstract

This thesis describes the use of narrow bore liquid chromatography (LC) combined with electrospray ionization (ESI) and tandem mass spectrometry (MS/MS) for determination of oxysterols in limited cell samples.

Paper I describes a validated method for determination of side-chain hydroxylated oxysterols (24S-hydroxycholesterol (24S-OHC), 25-hydroxycholesterol (25-OHC) and 22S-hydroxycholesterol (22S-OHC)) in cell samples using microbore LC-ESI-MS/MS. The oxysterols were derivatized into Girard T derivatives before separation on a 1 mm inner diameter (ID) reversed phase column. Sample clean-up was performed on-line using a robust automatic filtration/filter back-flush (AFFL) solid phase extraction (SPE) system. This system reduces manual handling of the sample and hence reduces the time consumption per sample and increase the repeatability. The positively charged Girard T derivatives were detected by positive ESI-MS. The AFFL-SPE system allows high volume injections (100 μ L) providing relatively good detection limits (<0.06 nM or 2.5 pg/6 fmol injected on column) with good precision. Autoxidation monitoring was enabled by adding isotope labeled cholesterol to samples and standards. Recovery in spiked cell lysate was between 105 and 109 %.

The method was used to identify and quantify side-chain hydroxylated oxysterols in cell samples from human colon cancer and immortalized mouse fibroblast cell lines. Two cell subpopulations from a human pancreatic cancer cell line (330 000 cells per population) were compared. The vimentin positive cell subpopulation (with increased tumor initiating potential) showed clearly higher levels of 24S-OHC compared to that of the vimentin negative subpopulation.

In **Paper II**, a high sensitivity and robust method for determination of side-chain hydroxylated oxysterols in limited cell samples is presented. Using nano LC (0.1 mm ID column) and a nano AFFL-SPE system, quantification of oxysterols (24S-OHC, 25-OHC and 27-hydroxycholesterol (27-OHC)) in only

10 000 pancreatic cancer cells was possible. The nano AFFL-SPE system allowed a relative large volume (5 μL) to be injected on the narrow column. In addition, the robust nano LC platform allowed hundreds of cell samples to be injected without column replacement. Quantification limits were 23 fg (65 amol) for 27-OHC and 25-OHC and 54 fg (135 amol) for 24S-OHC.

Although derivatization of oxysterol enables better detection limits than for native oxysterols in LC-ESI-MS/MS, a nano LC-ESI-MS/MS method for oxysterols without derivatization was pursued for reduced sample preparation (**Paper III**). Stable signals from oxysterol ions with loss of one or two water molecules were observed in single MS mode. In MS/MS mode the lack of charge retaining centers in native oxysterols created extensive fragmentation and difficulty to interpret mass spectra compared to that of the Girard derivatives. Extreme and persistent carry-over of native oxysterol on the narrow fused silica tubing (30 μm ID) used in nano LC made the combination not feasible.

To simplify the developed nano LC method (**Paper II**) and decrease the analysis time, and hence increase sample throughput, a method utilizing a capillary LC (cap LC) column (0.3 mm ID) was developed and validated (**Paper IV**). The AFFL-SPE-cap LC platform combines the robustness of the method in **Paper I** with the sensitivity of the method in **Paper II**. Using the AFFL-SPE system, 100 μL of derivatized sample could be injected in a reasonable time. The method was sensitive enough to detect oxysterols in limited exosomes samples from breast cancer cell lines and non-cancer cells. 27-OHC was highly enriched in the exosomes from an estrogen receptor positive (ER+) cell line compared to exosomes from estrogen negative (ER-), human embryonic kidney cell lines and human pooled serum.

With the methods developed in this thesis, highly sensitivity determination of cancer related side-chain hydroxylated oxysterols in limited cell and exosome samples is possible. These methods can be used to further examine the potential role of e.g. 27-OHC as a biomarker for ER+ breast cancer.

List of abbreviation and definitions

20 α -OHC	20 α -hydroxycholesterol
22S-OHC	22S-hydroxycholesterol
24S-OHC	24S-hydroxycholesterol
25-OHC	25-hydroxycholesterol
27-OHC	27-hydroxycholesterol
ACN	Acetonitrile
AFFL	Automatic filtration and filter backflush
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
cap LC	Capillary LC, Column ID 0.1-0.3 mm
CH25H	Cholesterol-25-hydroxylase
CID	Collision induced dissociation
CRD	Cysteine rich extra cellular domain
CRM	Consecutive reaction monitoring
CYP27A1	Sterol-26-hydroxylase
CYP46A1	Cholesterol-24-hydroxylase
EI	Electron ionization
EMT	Epithelial to mesenchymal transition
ER	Estrogen receptor
ER-	Estrogen receptor
ER+	Estrogen receptor positive
ESI	Electrospray ionization
FA	Formic acid
FDA	Food and Drug administration
GC	Gas Chromatography
GLI	Glioma-associated oncogene

HCD	High-energy collisional dissociation
Hh	Hedgehog
ID	Inner diameter
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
LXR	Liver X receptor
m/z	Mass-to-charge ration
MeOH	Methanol
micro LC	Column ID 1 mm
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
nano LC	LC using column ID < 0.1 mm
NMR	Nuclear magnetic resonance
PTCH	Patched
QqQ	Triple quadrupole
SERM	Selective estrogen receptor modulator
SIM	Selected ion monitoring
SMO	Smoothened
SPE	Solid phase extraction
SRM	Selected reaction monitoring
SUFU	Suppressor of fused
TIC	Total ion current
WB	Western blot

1 Introduction

1.1 Bioanalysis, diagnostics and cancer

Cancer is a heterogeneous group of diseases that features abnormal cell division (proliferation), but also the ability to promote malignancy in other tissues/organs (metastasis). In 2012 about 1.7 million women were diagnosed with breast cancer [1] and in 2013 over 30 000 were diagnosed with cancer in Norway [2], over 3 000 with breast cancer.

Analytical chemistry plays an important part in diagnostics of cancer. However, some of today's diagnostic methods are often based on medical imaging, e.g. mammography that can lead to missed, incorrect or delayed diagnosis in as much as 5 % – 15 % of the patients [3, 4]. By identification of new markers for diseases using unbiased analysis methods, this number could be reduced.

To handle the complexity of cancer, the treatment trends are pointing towards a more personal treatment (personalized medicine) [5], e.g. targeting estrogen receptor (ER) in hormone sensitive breast cancer or genetic testing for mutations in genes such as BRCA1 and BRCA2 [6]. Analytical chemistry will then play an important role in monitoring the progress of treatment and disease for each patient [5]. To do this, however, today's analytical tools must be improved to handle increased numbers of samples for high throughput analyses, with more automation and high method robustness, without compromising sensitivity and accuracy.

Metabolomics is the study of the metabolites present in cells, tissue and organs [7, 8]. In other words, measuring metabolites can give information about a process that has occurred place inside the cells; in comparison to measure DNA, RNA or proteins, which measures the process that might occur [9-11]. Increased or decreased production of a special metabolite can be used as a marker (biomarker) for e.g. progression in a disease [7-9]. Examples of metabolites can be sugars, vitamins and lipids. Metabolites can be determined by nuclear magnetic resonance (NMR), a quantitative and non-destructive

technique [7], however this technique is not very sensitive [10] (μM range sensitivity [7, 9]). A more sensitive method is to use mass spectrometry (MS) [11].

1.2 Liquid chromatography mass spectrometry (LC-MS)

Chromatography, in combination with MS, is a natural choice of analytical method when analysing complex samples, e.g. biological matrices such as blood and cells, especially when measuring metabolites. In chromatography (**Figure 1**) different components in a sample are separated in a column filled with a stationary phase. Depending on the physical properties of the analyte, the chromatography step can either be gas chromatography (GC, where the mobile phase is a gas) or liquid chromatography (LC, where the mobile phase is a liquid). Component in the sample will be separated according to their interaction with the stationary phase. LC is often the choice when analysing biological samples, such as peptides and steroids, as these analytes are not volatile enough for GC determination. In combination with MS, analytes can be identified and quantified based on both retention time (the time the analytes use through the column) and mass-to-charge ratio (m/z).

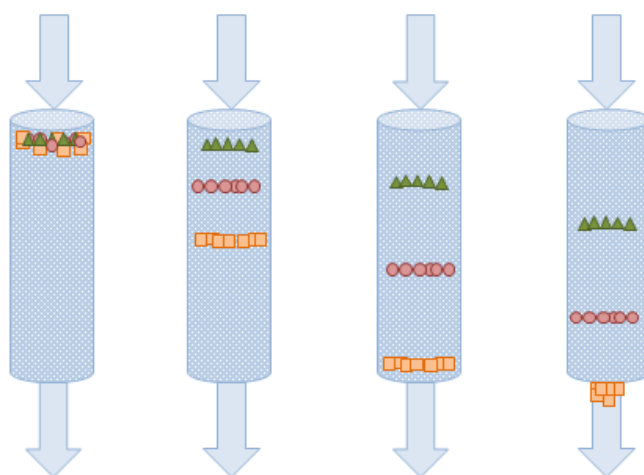


Figure 1 Separation of three different components on a column. Green interact most with the stationary phase, hence will use longer time (have longer retention time) through the column compared to orange, which has little or no retention. Red has medium interaction with the stationary phase, eluting in the middle and the components are separated.

ESI, which is a concentration dependent, soft ionization technique, brings ions from liquid to gas phase ions without extensive fragmentation. Hence ESI is more suitable for biomolecules compared to electron ionization (EI) used in GC-MS [12] as EI can cause extensive fragmentation hence loss of molecular mass information.

Determination of low abundant analytes in limited samples (e.g. exosomes or biopsies) can be challenging. The method of choice must be sensitive enough to detect the low abundant analytes against a complex matrix background. To gain high sensitivity with concentration sensitive detectors (e.g. ESI-MS), narrow LC columns can be applied [13-15]. By decreasing the ID of the LC column, less radial dilution of analyte band occurs, and hence a more concentrated analyte band elutes from the column [13, 14]. Chromatographic dilution, D , can be described by the formula

$$D = \frac{C_0}{C_{max}} = \frac{\epsilon \pi r^2 (1 + k) \sqrt{2\pi L H}}{V_{inj}}$$

where C_0 is the original concentration in the sample while C_{max} is the final concentration at the peak maximum, ϵ is the column porosity, r is the column radius, L is the column length, H is the column plate height and V_{inj} is the sample injection volume. Hence D will increase proportionally with the square of the column radius under the same chromatographic conditions. Consequently, a higher signal is measured if the same amount of analyte is injected on a narrow ID column compared to a larger ID column, as illustrated in **Figure 2**.

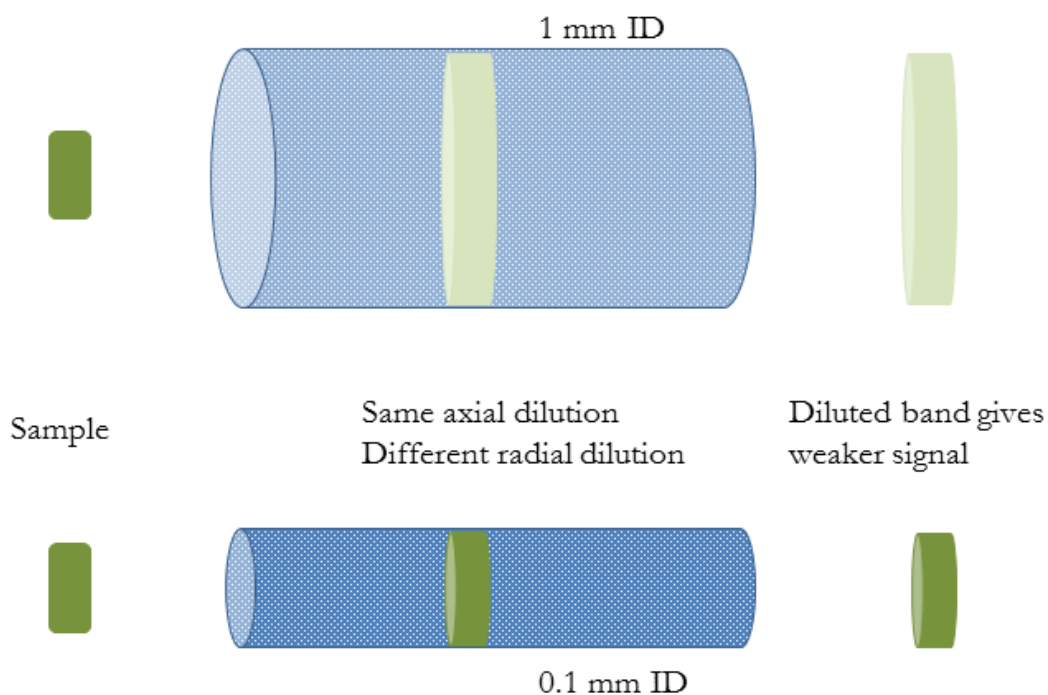


Figure 2 Illustration of band dilution on a micro (1.0 mm ID) and nano (0.1 mm ID) LC column. A narrower ID gives less radial dilution, and hence a more concentrated band elutes at the end (100x compared to larger ID). When using a concentration sensitive detector this will give a higher signal.

In addition, the transfer of analyte ions from liquid to gas phase ions using ESI is more efficient when using a lower flow of mobile phase, as needed with narrow LC columns [13, 16, 17]. Smaller droplets are generated and less solvent needs to evaporate, making the process from ions in liquid to ions in gas phase more efficient and higher sensitivity can be achieved. Narrow LC can be divided into several categories; microbore, capillary and nano LC, based on column dimensions and flow (**Table 1**).

Table 1 Narrow LC column dimensions, typical flow rate and injection volumes.

Name	ID (mm)	Flow rate	Calculated injection volume [13]
Microbore	1	50 $\mu\text{L}/\text{min}$	400 nL
Capillary	0.3	4000 nL/min	36 nL
Nano	<0.1	<500 nL/min	2.3 nL

One major challenge with cap LC and nano LC is the low injection volume these columns can handle [13-15, 17] (**Paper SIII**). Larger volumes of samples (with low elution strength solvent) can be injected on cap LC and nano LC columns; however this is a tedious process [13, 14] (e.g. injection of a 5 μL sample on a 0.1 mm ID column using a flow rate of 0.5 $\mu\text{L}/\text{min}$ would take at least 10 minutes). If phase focusing is not achieved the components in these samples will elute as broad peaks (**Figure 3**). Instead, column switching techniques are often used to increase the injection volume in narrow column LC [13-15](**Paper SIII**). A larger sample volume (with non-eluting solvent) can be focused on a SPE column using a high loading flow of non-eluting mobile phase. Subsequently, the analytes are eluted from the SPE and on to the analytical column for separation using a separate pump. A schematic view of a traditional column switching system using a 6 port valve is shown in **Figure 4**.

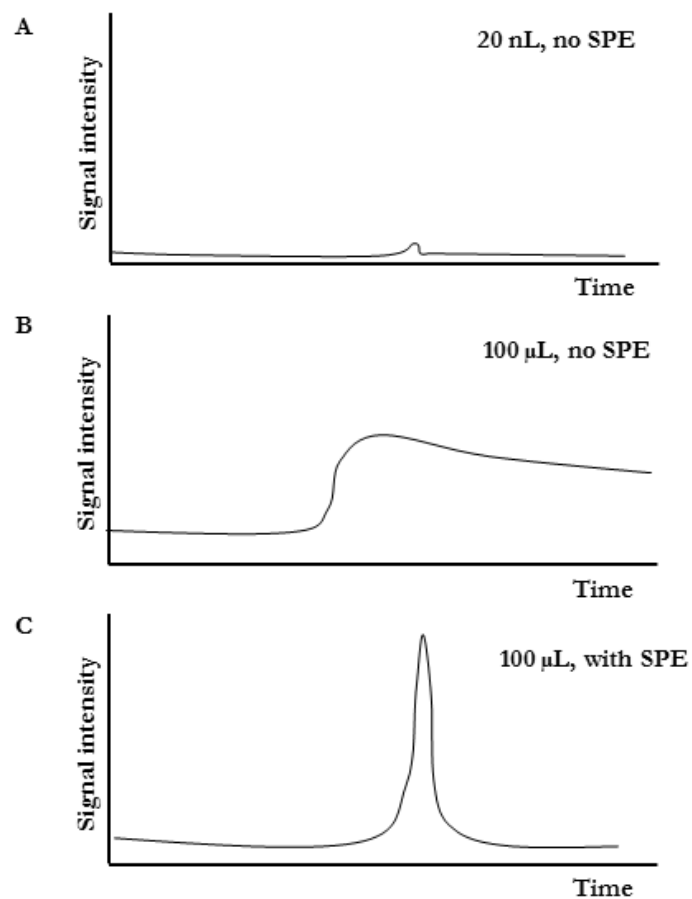


Figure 3 Illustration of injection on a nano LC column. A) Injection with too low concentration/volume. B) Injection of a too large volume giving extensive band broadening. C) injection of a high volume of low concentration sample using an on-line SPE-LC system (column switching system).

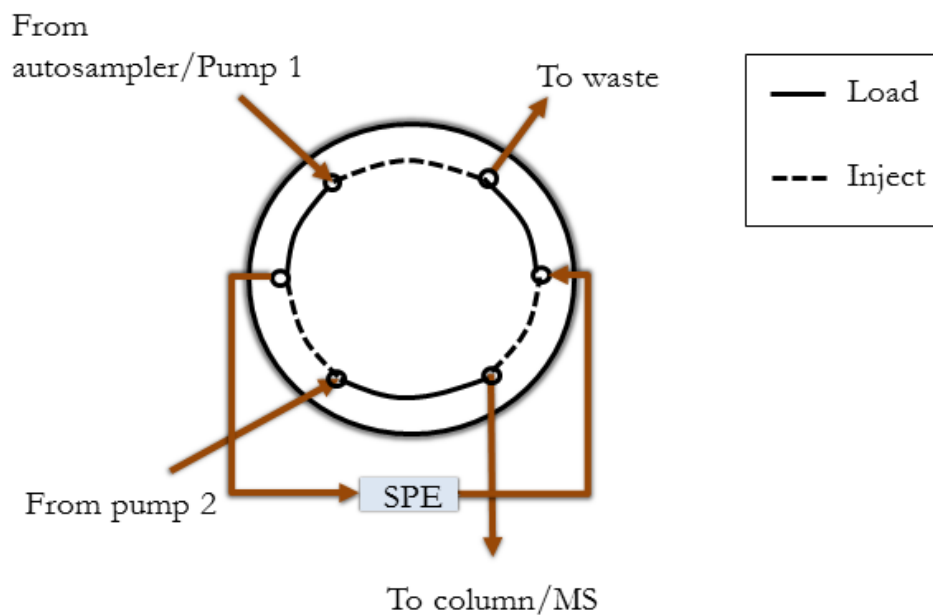


Figure 4 A traditional column switching set-up. In load position the sample is loaded on to the SPE, where analytes are retained while non-retained components and solvent are washed out to waste. When the valve is switched to inject position, a second pump elutes analytes in a narrow band from the SPE and on to the analytical column for separation. Valve set-up is shown as front-flush.

Unfortunately, column switching as a routine analysis technique has a bad (but deserved) reputation, as they are prone to pressure build up and clogging [12, 18](**Paper I, Paper SI**). An in-house developed technique, Automatic Filtration and filter back-Flush (AFFL) (**Figure 5**) has been shown to remove these clogging issues (**Paper SI**), hence making column switching a robust technique.

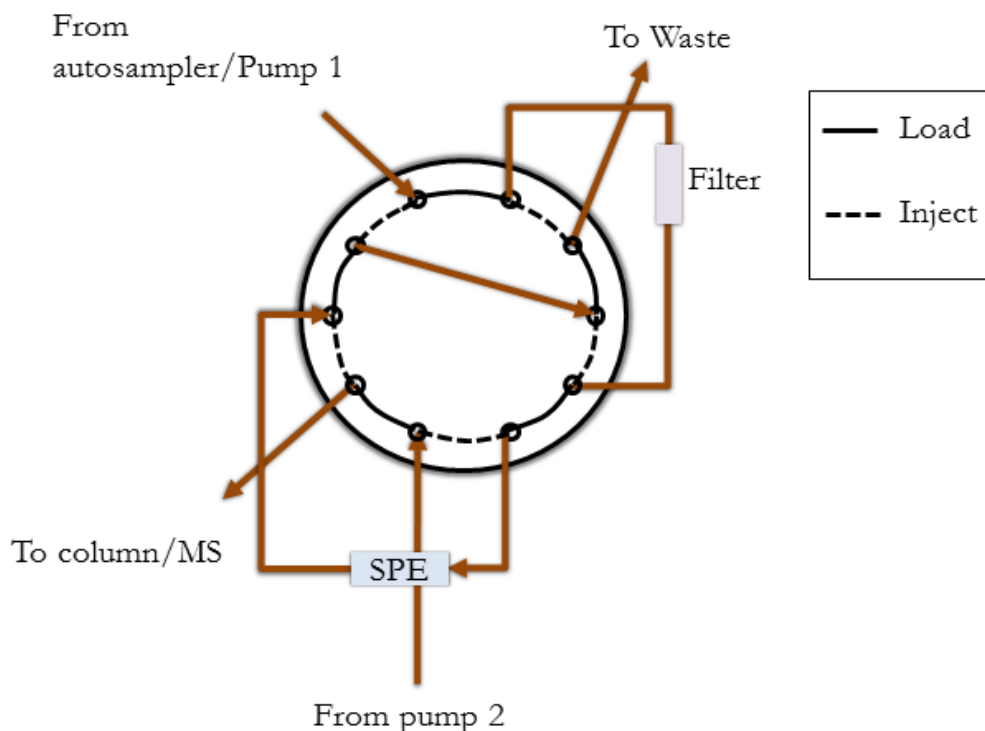


Figure 5 AFFL-SPE set-up. In load position, the sample is loaded through a filter and further on to the SPE. Particles are stopped by the filter, analytes are retained by the SPE, while non-retained components and solvent are washed out to waste. In inject position two things happen simultaneously: The analytes are eluted from the SPE in a narrow band and transported to the analytical column for separation. The pump used to load sample back-flushes the filter, removing particles from it and making the system clean and ready for the next sample. The set-up is shown in front-flush mode.

LC-MS can provide highly secure identification based on both the retention time and the m/z of the target analytes. Different MS instrumentation exists, e.g. quadrupole, ion trap and Orbitrap, with different advantages and disadvantages. One way of describing MS instruments are by resolution, which means the ability to distinguish two signals with slightly different m/z . Resolution, R is defined as:

$$R = \frac{m}{\Delta m}$$

where m is the mass of the ion of interest and Δm is the peak width. A higher resolution will make the instrument able to distinguish between two closely

m/z signals, e.g. separating analyte signal from matrix interferences signal. MS instrument can be operated in two scanning modes; total ion current (TIC) scans (all ions are monitored) or selected ion monitoring (SIM) scan (only selected m/z is monitored). A typical high resolution instrument is the orbitrap, while quadrupole is a typical low resolution instrument.

Some instruments, e.g. triple quadrupole (QqQ), hybrid orbitrap (e.g. Q Exactive™ = quadrupole orbitrap) and ion traps are able to perform MS/MS to gain higher selectivity and a more secure identification. In MS/MS, selected reaction monitoring mode (SRM) [19], precursor ions are selected based on m/z in the first MS, fragmented in a collision cell followed by identification of product ions in the second MS. Only ions with a given m/z that fragment in to specified m/z are measured. Background noise is reduced as fewer ions hit the detector and both lower limits of detection (LOD) and increased selectivity is achieved. Ion traps are able to perform multiple MS (MS^n) through consecutive reaction monitoring (CRM) [19]. With MS^n , more structure information about the analytes can be gained and used to identify unknown isomer structures [20].

This thesis will focus on the determination of oxysterols, using sensitive LC-MS with focus on automatic and robust methods. These oxidized cholesterol metabolites might play an important role in the Hedgehog (Hh) signaling pathway [21], but also in both proliferation and metastasis in ER positive breast cancer [22, 23].

1.3 Oxysterols

Oxysterols are small, neutral metabolites of cholesterol, formed either enzymatic or by autoxidation (also called non-enzymatic oxidation) both endogenously and during sample preparation [24-27]. These oxygenated 27-carbon cholesterol metabolites have a typical cholesterol based steroid structure [28] and pose multiple roles in the body, including cholesterol homeostasis [29-34] and signaling [27]. Cholesterol and some of the side-chain hydroxylated oxysterols are shown in **Figure 6**. Systematic names for all these oxysterols are shown in **Table 2**, however the trivial names will be used

throughout this thesis. 27-OHC will be used in this thesis, although the correct name would be 25R,26-hydroxycholesterol, as 27-OHC is more used in the context of medicine and cancer [35].

The enzyme sterol-27-hydroxylase (CYP27A1) is a part of the cytochrome P450 family and responsible for metabolising cholesterol into 27-OHC [24, 25, 34, 36]. The enzyme is expressed in macrophages and tissue [25, 27, 34, 35]. 27-OHC has multiple biological roles, such as selective estrogen receptor α modulator (SERM) [37, 38] and liver X receptor (LXR) agonist [39] and has recently been closely connected to ER+ breast cancer [22, 23, 40]. 27-OHC is also an intermediate in the bile acid production where the CYP27A1 enzyme converts cholesterol into 27-OHC and further in to 27-cholestenoic acid [38, 40-42].

Cholesterol-24-hydroxylase (CYP46A1) is mostly found in the brain and converts cholesterol into 24S-OHC (also known as cerebrosterol) [25, 26, 31, 34, 36]. More specific, this enzyme is located in the mitochondria of the cells [36, 43] and in endoplasmic reticulum [27, 36]. The enzyme is important in the major pathway for brain cholesterol homeostasis maintenance [24-26, 44], as 24S-OHC is able to cross the blood-brain barrier, while cholesterol is not [24, 45].

Cholesterol is converted to 25-hydroxycholesterol (25-OHC) mainly by the enzyme cholesterol-25-hydroxylase (CH25H), however other sources might also exist [25, 31]. This enzyme, unlike CYP46A1 and CYP27A1 is not belonging to the cytochrome P450 family [25, 27, 36]. The function of 25-OHC was previously addressed to regulating lipid metabolism; however recent studies, together with its low concentration in tissues have rather pointed out this oxysterol as an immune regulator [24, 44].

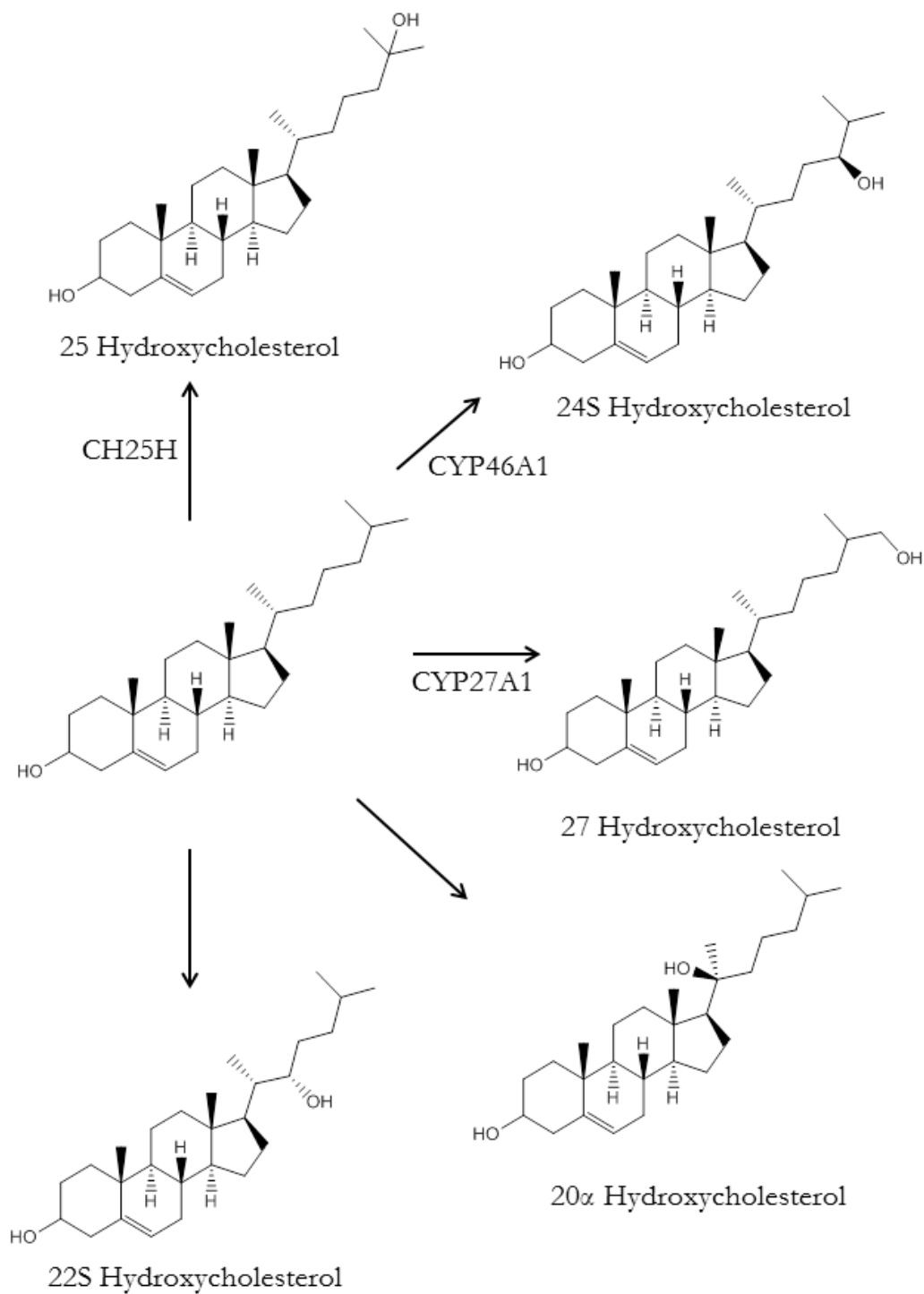


Figure 6 Side-chain hydroxylated oxysterols 25-OHC, 24S-OHC, 27-OHC, 20 α -OHC and 22S-OHC together with their precursor cholesterol and known enzymes.

22S-OHC and 20 α -hydroxycholesterol (20 α -OHC) are activators of the Hh signaling pathway *in vitro* [46-48], where the latter is more potent [21, 47]. To

the author's knowledge these oxysterols are not naturally formed in the body, as no known enzyme exist. 20α -OHC has not been identified endogenously in samples in recent studies.

Table 2 Trivial and systematic name of oxysterols

Trivial name	Systematic name
24S-hydroxycholesterol (Cerebrosterols)	Cholest-5-ene- 3β ,24S-diol
25-hydroxycholesterol	Cholest-5-ene- 3β ,25-diol
22S-hydroxycholesterol	Cholest-5-ene- 3β ,22S-diol
20α -hydroxycholesterol	Cholest-5-ene- 3β ,20R/S-diol
27-hydrocholesterol	Cholest-5-ene- 3β ,26-diol

1.4 Biological role of oxysterols

Different oxysterol isomers are involved in different biological roles including cholesterol homeostasis [24, 34, 49], calcium uptake and apoptosis [50], atherosclerotic plaque formation [27, 34] in addition to being a biomarker for Niemann-Pick type C1[51]. In most cases, both the position of the hydroxyl group and stereochemistry (3β -hydroxyl group of cholesterol [52]) determine activity (e.g. in Hh signaling pathway [21, 47], LXR [34, 52] and ER [38]). The biological role of oxysterols with implications to this thesis is briefly described below.

1.4.1 Oxysterols and the Hedgehog signaling pathway

The Hh signaling pathway is important in development of embryos and regeneration of human adult stem cells [21, 53, 54] (**Paper SII**). However, an overactive signaling pathway is highly associated with cancer [46], in particular medulloblastoma [55] and basal cell carcinoma [56]. In general, approximately 30 % of cancers have elevated Hh signaling [57], hence making it a target for cancer drug development [54]. The first drug (Vismodegib) targeting this

pathway was approved by the food and drug administration (FDA) in 2012 [58].

The Hh signaling pathway has two central proteins, patched (PTCH) and smoothened (SMO). In the off state (**Figure 7**) of the signaling pathway, PTCH inhibits SMO to enter the primary cilium of the cell. Glioma-associated oncogene (GLI) complexes with suppressor of fused (SUFU) in to a large protein complex and no transcription occurs [46, 47, 54, 59]. When an Hh protein with a cholesterol modification at the C- terminus is delivered to PTCH [48, 55], e.g. by exosomes [53], SMO is released, phosphorylated and enters the primary cilium [21]. GLI3 proteins are released from SUFU and enter the nucleus and the transcription of genes *GLI1*, *GLI2* and *GLI3* occurs.

The role of oxysterols and other steroids in the Hh pathway is not fully understood [21, 48, 55]. Hh signaling is activated by oxysterols *in vitro*, in particular by 20 α -OHC [21, 46, 48, 55, 60]; however the other endogenously formed oxysterols can activate the pathway as well [46, 48, 55, 61], by binding to the cysteine rich extracellular domain (CRD) of SMO [47, 48]. *In vivo* activation of the Hh signaling pathway by specific endogenous small molecules ligands of SMO (e.g. oxysterols) is, however, still debated [55].

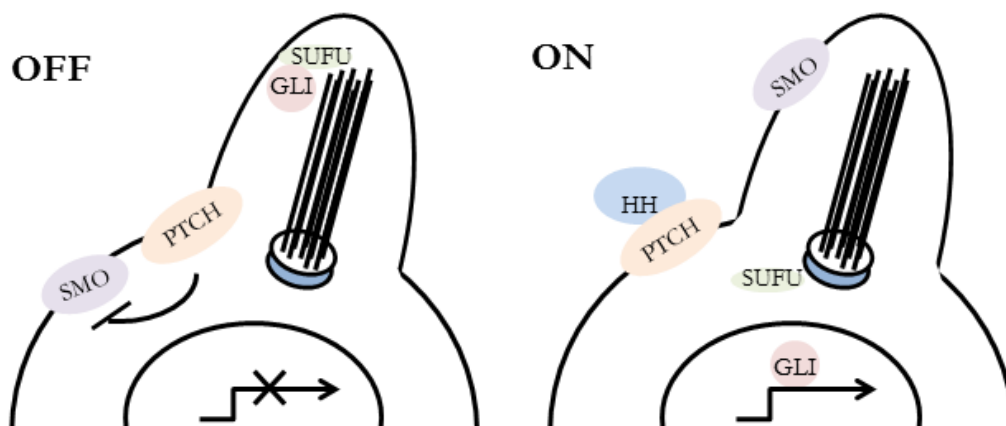


Figure 7 The Hh signaling pathway in OFF and ON state. In OFF state PTCH inhibits SMO and no gene transcription occurs. In the ON state, a Hh protein binds to PTCH and transcription of genes occurs

1.4.2 Oxysterols as activators of nuclear receptors

Nuclear receptors are ligand activated transcription factors, which regulates homeostatic biological processes [29, 62]. These receptors are often sterol sensing receptors [29, 30, 62] and in the human genome there are 48 members of this transcription factor family [62]. The nuclear receptors can be divided into two subgroups; classic nuclear steroid hormone receptors such as ER, androgen receptor, glucocorticoids, mineral corticoids and progesterone receptors [62]. The other subgroup contains the orphan receptor (with unknown endogen ligands), where also “adopted” orphan receptors are included (endogen ligand identified)[62]. Oxysterols can activate ER receptors (27-OHC) [22, 23, 38, 63] and are also endogenous activators of an adopted orphan receptor, LXR [26, 29, 30, 34, 52, 62].

1.4.2.1 Oxysterols and estrogen receptor

Estrogen receptors play a major role in ER+ breast cancer development, and an important way to treat breast cancer is by ER blockers [64-68], such as tamoxifen and letrozole [6, 68, 69]. ER can be classified into two subgroups, ER α and ER β . These two subgroups differ in ligand binding, expression pattern and biological activity [38, 68]. ER α is the main target in ER+ breast cancer, due to increased protein levels in premalignant and malignant lesion compared to normal breast tissue [65].

SERM can act as both estrogen agonist and antagonist depending upon the target organ [37, 39, 64, 69]. 27-OHC is classified as a endogenous SERM that modulates ER function [39, 69] (**Paper SIV**). 27-OHC can block the effect of 17 β -estradiol (E2) [38, 40] and in breast cancer, 27-OHC can regulate ER α transcriptional activity [38]. Treatment of MCF-7 cells with 27-OHC, an ER+ breast cancer model cell line, leads to proliferation [22, 23, 38, 40].

1.4.2.2 Oxysterols and Liver X receptors

The LXR are two nuclear receptors (LXR α and LXR β) which control lipid and cholesterol metabolism [29-32]. LXR α is mostly expressed in metabolic active tissues such as liver, kidney, adipose tissue and macrophages, while LXR β is

expressed in all kind of tissue [32, 62] and especially in developmental brain [30]. LXR activates genes such as transport, catabolism and elimination of cholesterol [26, 62] and one important target gene is the CYP7A1 gene, which catalyze the rate-limiting step in bile acid production [30, 32, 62, 70]. LXR is activated by endogenous levels of specific oxysterols (22R-OHC, 24S-OHC, 24S,25-epoxycholesterol and 27-OHC) [29, 30, 32, 52, 62, 70], hence acting like a cholesterol sensor inside the body [30, 32]. When the cholesterol content is increased, more oxysterols are formed and activate the LXR pathway, which again removes cholesterol by turning it into bile or bile acid [30, 32, 62].

1.5 Determination of oxysterols

Determination of oxysterols and other steroids can be challenging as these molecules are not volatile enough for GC determination and are not easily ionized with ESI, the most common ionization technique for LC-MS. Some groups have solved this by e.g. measuring mRNA expression of the enzyme [22] creating the oxysterol by real time quantitative PCR, although transcription, protein levels and metabolite concentrations in general not always correlate [11, 71-74](**Paper II, Paper IV**).

Another alternative for determination of the oxysterol is to derivatize the oxysterols, either to enhance the GC performance (e.g. with trimethylsilanol) [28, 75, 76], or to enhance ionization efficiency in ESI (e.g. with Girard reagents [20, 24, 26, 28, 34, 77, 78]). The detection limits for side-chain hydroxylated oxysterols achieved with different strategies/methods for oxysterol determination are shown in **Table 3**, including approaches as using atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI). These two ionization modes are mass sensitive and only compatible with high flow LC (>0.5 mL/min). Lowest limits of detection (LOD) are achieved with LC-ESI-MS with derivatization which increases ionization efficiency.

Table 3 Detection limits published for oxysterol determination.

Method	Derivatization	Method detection (MD) ^{AB*} (pg)	LOD ^C /LOQ ^D (ng/mL)	Source
GC-EI-MS	TMS	700 - 3 000 ^A	700 – 3 000 ^{E*}	[75]
GC-CI-MS/MS	TMS	0.05-0.23 ^{A*}	0.05-0.23 ^C	[76]
LC-ESI-MS/MS	Picolinyl	0.005-0.01 ^A	0.005-0.01 ^{C*}	[70]
LC-ESI-MS/MS	Girard P	1.5 ^A	0.15 ^{C*}	[77]
LC-ESI-MS/MS	Girard T	0.023-0.054 ^A	0.005-0.01 ^C	Paper II
LC-ESI-MS/MS	N,N-dimethylglycine (DMG)	5-22 ^{B*}	1-4.5 ^D	[33]
LC-ESI-MS	No (with adduct formation)	50 ^A	1 ^{C*}	[44]
LC-APPI-MS	No	12.5-125 ^{A*}	2.5 ^C	[79]
LC-APCI-MS/MS	No	20 ^{B*}	1 ^D	[80]
LC-APCI-MS	No	150-500 ^{A*}	1.2-1.5 ^C	[49]

^A LOD is used.

^B LOQ is used, LOD not published.

* Calculated based on LOD/LOQ and injection volume.

^C LOD

^D LOQ

^E Assuming 1 μ L injection volume

1.5.1 Derivatization with Girard reagent

Derivatization of sterols with Girard reagents has been performed for decades [81], however as a method of sample preparation for enhanced ionization in oxysterol determination, this method was established by Griffiths et al [77]. The derivatization is performed in two steps: Firstly, the 3 β -hydroxy group is oxidized into a carbonyl group using an enzyme, cholesterol oxidase. Secondly,

the carbonyl group is reacted with a Girard reagent and a quaternary amine is included into the structure. The reaction is shown in **Figure 8**, and the ionization efficiency is enhanced 2-3 orders of magnitude [28, 77]. Two different Girard reagents; Girard P and Girard T (**Figure 9**) are available. In our hands, the Girard T reagent provided better chromatographic performance, and was therefore chosen.

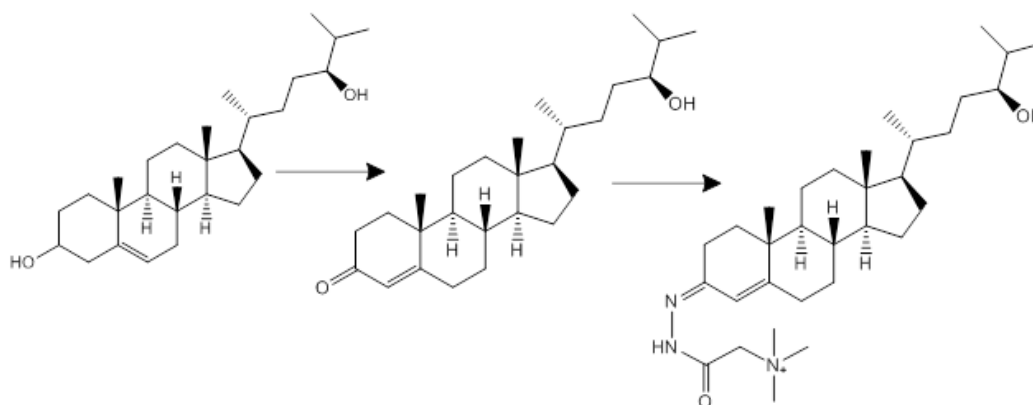


Figure 8 Derivatization of oxysterols into Girard T derivatives. Firstly, the 3 β -hydroxygroup is oxidised. Subsequently, the formed carbonyl group is reacted with Girard T reagent.

One benefit of derivatization of oxysterols with Girard reagents is the more specific fragments created when using MS/MS [20, 82] (**Paper III**). Girard T derivatives of the oxysterols give an intense $[M]^+$ ions in MS mode and fragment in MS/MS $[M]^+ \rightarrow [M-59]^+$. Native oxysterols often loses one or two water molecules in MS mode. In MS/MS mode, native oxysterols fragments into many ions in the low m/z range. Further, by employing MS³/ion trap instruments on Girard derivatives, some structure information is possible to identify by fragmentation of the product ions [20].

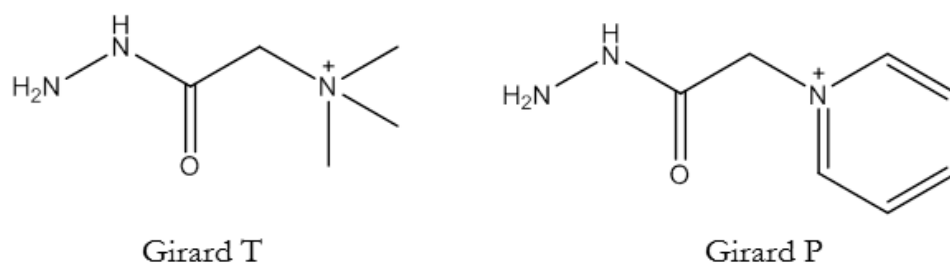


Figure 9 Girard T and Girard P reagent

One drawback with the Girard derivatization is that the reaction is not stereospecific; hence *syn* (or *Z*) and *anti* (or *E*) isomers are introduced (**Figure 10**). This might give rise to two chromatographically separated compounds for each oxysterol using LC-MS [28, 70].

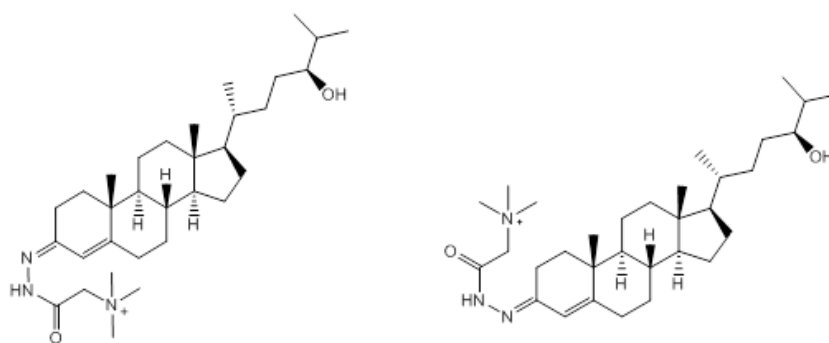


Figure 10 *Syn* (*Z*) and *anti* (*E*) forms of Girard T derivate of 25 hydroxycholesterol

A potential pitfall using the Girard derivatization is that the sample preparation method will not distinguish between natural occurring 3-keto groups and those groups created by the cholesterol oxidase enzyme [24, 70] (**Paper II**). This can potentially give false positive results. The easiest way to examine this is to split samples into two subsamples, where one subsample is subjected to full sample preparation, included cholesterol oxidase, while the other subsample is not subjected to oxidation with cholesterol oxidase. Hence the second subsample can be used to identify oxysterols with natural occurring

3-keto groups and thus correct the quantification of oxysterols without natural occurring 3-keto group [24] (**Paper II**).

An additional aspect of derivatization with Girard reagent, compared to some of the other published derivatization methods for LC and GC, is the low temperature needed for reaction (oxidation at 37 °C and Girard reaction at room temperature in the dark). Low temperature is wanted as cholesterol in the sample can autoxidize into oxysterols during sample preparation when the sample is subjected to heat or light [25, 83, 84]. Even a small extent of autoxidation of oxysterols during sample preparation can give elevated concentration of target analytes, hence false positive results [24, 27, 49]. Several measures can be included in the sample preparation to avoid autoxidation, e.g. removal of cholesterol from the sample [26], adding antioxidants such as butylated hydroxytoluene (BHT) or purging all vials with Ar [24]. This might be especially important if the target analyte is $7\alpha/\beta$ /keto-hydroxycholesterol, as the 7th position is more prone to autoxidation [24-27, 83].

To monitor for autoxidation isotope labeled cholesterol can be added to samples before sample preparation (e.g. $^2\text{H}_7$ -cholesterol or $^{25,26,27-13}\text{C}_3$ cholesterol) [26] (**Paper I, Paper II, Paper IV**). This action will not prevent autoxidation, but can be used as a marker to confirm if autoxidation has occurred. If natural occurring cholesterol is oxidized into oxysterols, isotope labeled cholesterol is oxidized into isotope labeled oxysterols. This can be monitored for using MS.

2 Aim of Study

To discover new diagnostic markers in cancer, hence get a deeper understanding of cancer, cancer cell subpopulations must be investigated. Challenges with limited available samples from e.g. slow cycling cancer cells with stem cell like traits, biopsies and exosomes, must be met with high sensitivity and reliable robust analytical methods. Oxysterols are closely related to the Hh signaling pathway and ER+ breast cancer; however, complete role is not understood. To further investigate the role of oxysterols in relation to cancer and in particular breast cancer, analytical methods for determination of oxysterols must be developed and used for cancer research.

The aim of this study was threefold. Firstly, an automated and robust method for determination of oxysterols in cell samples should be developed. This method should secondly be sensitive, so that oxysterols could be determined in limited samples, such as cell subpopulations, without compromising robustness. Thirdly, the method should be as simple as possible, allowing both relative high throughput and still keep the robustness and sensitivity achieved with the first two goals.

3 Results and Discussion

To meet the aims of this thesis, a method for determination of oxysterols in cell samples, which included robust on-line sample clean-up for removal of derivatization reagents, was developed (**Paper I**). The method was further made ultra-sensitive by downscaling; resulting in a robust nano LC platform (**Paper II**). To simplify the sample preparation method, an attempt to develop a nano LC method for native oxysterols was pursued, however this turned out to be unsuccessful (**Paper III**). All the experience gained in **Paper I-II** were combined to develop a cap LC platform for determination of oxysterol in cancer exosomes in **Paper IV**. In the following, the method development will firstly be discussed, and subsequently the biological results.

3.1 Method development

An analytical method for biological samples should be sensitive and robust, in addition to be selective for the analytes. By combining LC and MS, detection can be made more selective and un-biased compared to e.g. Western blot (WB), which is based on visual inspection of protein bands. Also, by measuring the metabolite itself (e.g. oxysterols), a better biological understanding of cancer progress, e.g. in combination with measuring proteins, can be made. Automatization of necessary sample clean-up, e.g. by AFFL-SPE, gives less manual handling of the sample, hence removing potential personal errors in sample handling.

3.1.1 Automatic filtration and filter back-flush

Derivatization reactions, e.g. with Girard reagents, requires a sample clean-up before introduction into the LC-MS system to avoid possible matrix effects [12, 16, 28, 85] or clogging of the system. The sample clean-up can be manual, (off-line), or automatic (on-line), performed by the LC-MS system e.g. by using a column switching system. The main focus in **Paper I** was to develop a robust column switching system for on-line sample clean-up of samples for the not easily removed Girard reagents that can cause ion suppression in the ESI [28]. The system should allow for many large volume cell sample

injections without the need of continuous maintenance as often needed when using a traditional column switching system (**Paper SI**). Column switching systems are required to inject large volume of sample onto narrow LC columns, without column overloading and/or extensive band broadening [15, 86]. A narrow LC column was chosen as the method was to be used for limited cancer cell subpopulations. With the use of a 10 port switching valve, our in-house developed AFFL-SPE system (**Paper SI**) was used, with a micro bore (1 mm ID) analytical column (**Paper I**). This allowed repeatable injections without the common pressure build up that usually arise when samples are clogging up the SPE column [12, 18] (**Paper I, Paper SI**).

By including the AFFL-SPE system together with an “in one vial sample preparation” and without removal of cell debris and protein precipitation, analyses of limited cancer cell samples were possible (**Paper I, Paper II, Paper IV**). Protein precipitation and particles that can clog the SPE are stopped in the filter and back-flushed off the filter after injection. With an “in one vial sample preparation”, there is no additional transfer of sample between vials, and the potential loss of analytes is avoided.

The power of this AFFL-SPE system is shown in **Figure 11 (Paper I)**, where pressure over the SPE is compared with and without the AFFL system in front. After only 10 injections of derivatized cell lysate without the AFFL system, the pressure on the SPE was doubled, and after only 40 sample injections, the SPE was clogged and needed replacement. The AFFL-SPE system allowed thousands of injections without SPE replacement (**Paper I**).

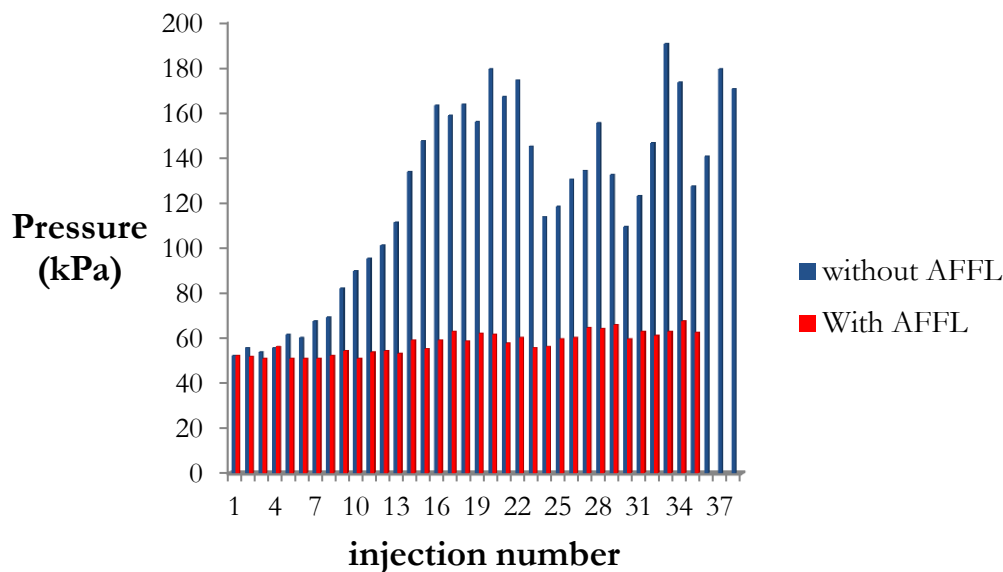


Figure 11 Comparison of pressure on SPE column with and without the AFFL system (Paper I).

3.1.2 Ultra-high sensitivity measurement of oxysterols

Utilizing the experience with AFFL-SPE in **Paper I**, the goal for **Paper II** was two-folded. Firstly, to be able to look at slow growing cancer cell subpopulations and other limited samples, the sensitivity needed to be increased. Secondly, a robust nano LC platform was pursued to show both the potential of nano LC in routine measurements, and that nano LC not only can be used for proteomics, but also for metabolomics/lipidomic.

To increase sensitivity, a nano AFFL-SPE-nano LC- MS system was used together with a high resolution MS (Q Exactive™ Orbitrap MS). Additionally, the column switching system was rebuilt/simplified from **Paper I** to **Paper II**, to use two pumps instead of three [87]. A comparison of the plumbings is shown in **Figure 12**. With the AFFL-SPE-nano LC system hundreds of sample injections could be performed without compromising chromatographic resolution. Also, method mass detection limits (MDs) in the low fg range were achieved, allowing detection of oxysterols in only 10 000 pancreatic cancer cells (**Figure 13**).

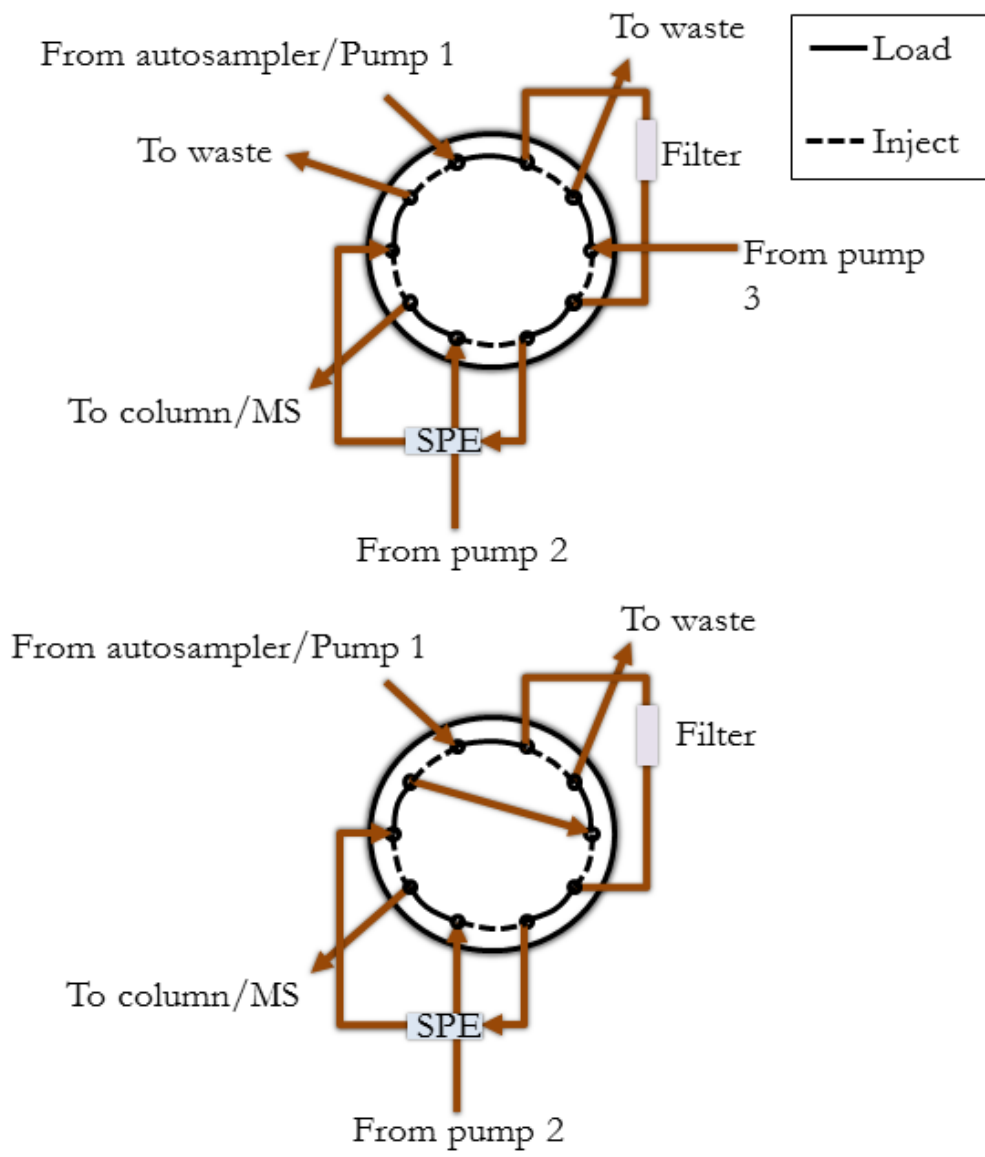


Figure 12 AFFL-SPE system with three pumps (top) and two pumps (bottom) set-up.

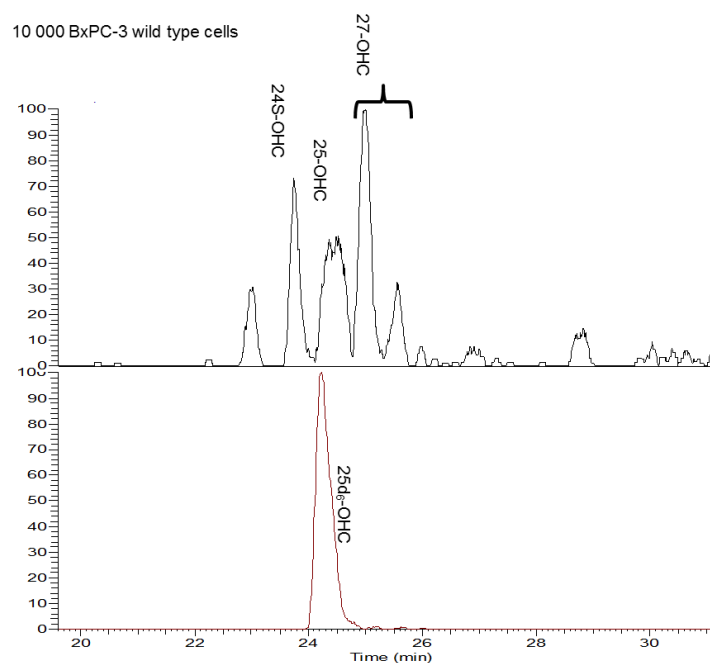


Figure 13 Extraceted ion chromatogram (EIC) of m/z 514.44→455.36 (top, analytes) and 520.40→461.40 (bottom, internal standard) of oxysterols in 10 000 BxPC-3 cells (Paper II). Derivatized cell samples was analysed using AFFL-SPE-nano LC MS/MS. Injection volume was 5 μ L and samples was loaded on the Hotsep Kromasil C₈ (0.3 mm ID x 5 mm) SPE column using 0.1 % formic acid (FA) in H₂O (flow rate 15 μ L/min). Subsequently, after 3.5 minutes, the valve was switch. Derivatized oxysterols were eluted off the SPE column and transferred to the analytical column (ACE 3 C₁₈, 0.1 mm ID x 100 mmm) for separation using FA/H₂O/Methanol (MeOH) (0.1/5/95 v/v/v %) with a flow rate of 0.5 μ L/min (isocratic conditions).

3.1.3 Challenges with the AFFL-SPE-nano LC system

The main challenge with the AFFL-SPE-nano LC system was, however, the introduction of extra column volumes from the 10 port valve. Even with the use of narrow tubing (ID \leq 30 μ m), the bore of the 10 port valve together with tubings created large extra column volumes. Although making couplings without dead volumes and maintaining a good peak shape was possible, the low flow through the large couplings gave increased analysis time per sample (total analysis time app. 75 min per sample, including sample loading and re-equilibration). To counteract the effects of the increased extra column volumes, narrower tubing was examined; however, loading of samples with increased flow was difficult due to the resulting high back-pressure in the narrow tubing. Hence, only 5 μ L sample was loaded with a loading flow rate of 15 μ L/min. If a larger sample volume could be loaded on to the SPE

column, better detection limits could be achieved, however this was not feasible. Decreased MDs is needed for either analysing smaller samples (e.g. exosomes, biopsy), or, by increasing the starting sample size (e.g. from 10 000 to 500 000 cells), explore the possible other oxysterols not present above detection limits of the method in limited 10 000 cell samples.

3.1.4 Further development of the nano LC method

Due to technical problems and expensive repeatable maintenance with the Agilent nano LC pump used in **Paper II**, the pump was replaced with a Proxeon nLC pump. The Proxeon nLC pump had a built in autosampler/sample loading configuration that was modified to an AFFL-SPE system as shown in **Figure 14**. This system was semi-validated by master student C Vesterdal and showed good precision and repeatability (data not published). The Proxeon nLC pump uses two syringe pumps to deliver flow rates at nL/min without flow splitting, while the Agilent pump splits the flow. Hence by using the Proxeon nLC pump, less solvent is used, and further gives environmental and economic benefits. One drawback of the Proxeon nLC pump is that it uses channel A syringe pump to load the sample, and the flow through the column is stopped during sample loading. While the pump prepares for the run, mobile phase A is pumped through the column, hence the pump is not re-equilibrated with isocratic separation conditions before sample injection. Thus the pump is not very suited for isocratic elution with high organic solvent content in the mobile phase. When using the isocratic separation conditions described in **Paper II**, broad peaks appeared and no separation was achieved. Adding MeOH to the mobile phase A reservoir to counteract these effects resulted in sample loading breakthrough, meaning that the analytes were washed off the SPE and to waste during sample loading. Since the Proxeon nLC pump is built for gradient elution, different gradients were examined to counteract the band broadening effects. The best separation with the AFFL-SPE-Proxeon nLC setup was achieved with a gradient from 70 % B to 90 % B in 20 minutes (data not published).

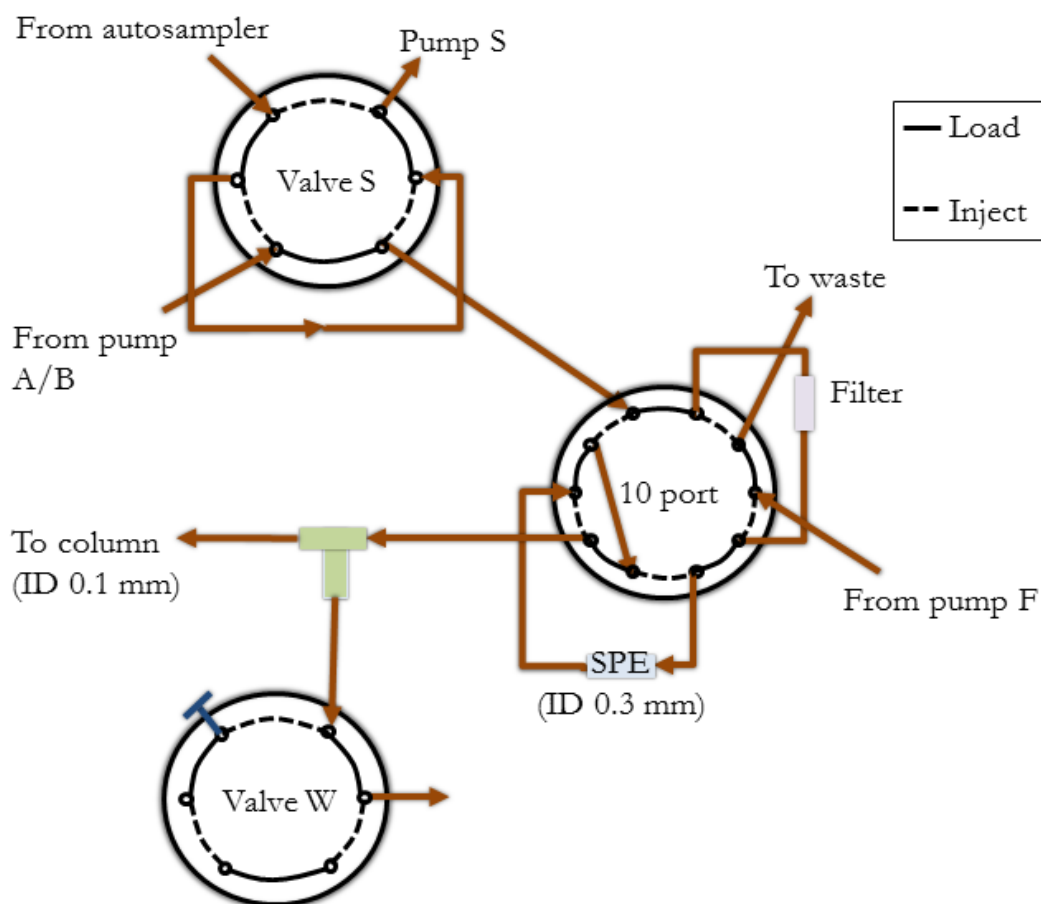


Figure 14 AFFL-Proxeon nLC set-up, utilizing the built-in sample loading system (valve S and valve W) and external AFFL-SPE 10 port valve. Sample is loaded with mobile phase pump A. Filter is backflushed with pump F.

3.1.5 Simplifying the methods for higher throughput

After derivatization of samples and standard solutions, the total sample/standard solution volume was 720 μL . This volume was difficult to reduce and still preserving analytical volumetric control of the reagents added. Using the nano LC platform, increasing the injected volume was difficult; hence to better exploit the total sample volume, a cap LC platform (column ID 0.3 mm) was built (**Paper IV**). The AFFL-SPE-cap LC platform allowed a higher injection volume (100 μL) to be injected without compromising chromatographic resolution. Actually, the chromatographic resolution was increased when using the cap LC platform compared to the nano LC platform running the same gradient as used on the Proxeon nLC set-up.

With the cap LC column (0.3 mm ID) radial dilution will increase by a factor of 9 compared to the 0.1 mm ID nano LC column, hence increasing the detection limits. Nonetheless, by increasing the injection volume from 5 μL to 100 μL , the same limits of quantification (LOQ) as obtained by nano LC was achieved. Theoretically, a 20x increase of the injection volume should have given even lower quantification limits, as the radial dilution only was increased by a factor of 9. Nevertheless, limit of detection (LOD defined as 3 x signal/noise ratio) was indeed lower for the cap LC system compared to the nano LC system. Possible breakthrough while loading 100 μL sample on a 0.3 mm ID SPE together with not ideal MS conditions (e.g. not clean enough MS) could be other reasons for not achieving better LOQ.

The higher flow rate used in cap LC (low $\mu\text{L}/\text{min}$) allowed for faster equilibration compared to nano LC (nL/min); hence the analysis time was reduced from app. 75 minutes per sample to 40 minutes per sample, including sample injection, sample loading and equilibration of column after sample.

Another possible reason for not achieving lower LOQ might be the ESI interface. With cap LC, a standard ESI was applied, while with nano LC, a nano ESI interface was used. Due to smaller droplets formed in nano ESI (lower flow), this technique gives more efficient ionization, although it is more technical challenging to work with.

The cap LC platform was easier to operate compared to the nano LC system. Troubleshooting was easier due to higher flow rates (e.g. easier to find leakage). Connecting the LC and MS was also easier, as the standard ESI source is more robust and easier in use compared to the nano ESI source. With nano ESI, the spray needle needs to be aligned perfect with the MS inlet to achieve stable nano spray. Using higher flow rates and a standard ESI source, the fixed position of the spray needle makes connection between LC and MS more reliable and does not demand daily adjustment, hence making the cap LC platform more robust compared to the nano LC platform. Taking all these

experiences together, the cap LC platform would be the recommended platform for determination of oxysterols in limited samples.

3.1.6 Effects of cholesterol on column

A common concern when determining oxysterols is excess cholesterol, both with regards to possible autoxidation during sample preparation, but also as cholesterol might compromise the chromatography. Cholesterol can change the separation properties of the column [88, 89], by making a pseudo stationary phase. Removal of cholesterol before derivatization of the oxysterols would require more extensive manual sample preparation, as this procedure most often requires a manual SPE step before derivatization. In our hands, while monitoring for autoxidation using isotope labeled cholesterol, autoxidation has not been an issue.

However, when using MeOH as mobile phase, complete elution of cholesterol from the SPE and the analytical column was not possible. Accumulation of cholesterol on the column was observed as peak broadening, hence loss of separation, followed by decreasing peak area as the analytes possibly adsorbed to the cholesterol in the column. These effects were easily removed by washing the column with acetonitrile (ACN). Nevertheless, these effects seemed to be more critical in nano LC determination of derivatized oxysterols, as the column required cleaning after approximately six samples (**Paper II**), while the cap LC column (**Paper IV**) was cleaned once every day.

3.1.7 Purity of standards and reagents

One challenge with ultra-sensitive determinations is the availability of pure standards. This was a challenge for the oxysterols, especially for 20 α -OHC. In method development for **Paper I**, several peaks with similar intensity were found for the 20 α -OHC standard, implying that the purity was not good. As shown by the ¹³C NMR measurement in **Paper I (Figure 15)**, the standard did indeed contain different isomers. 20 α -OHC standards from other vendors have been examined chromatographically, with the same results (data not

published). Accurate quantification of this particular oxysterol is therefore challenging due to lack of pure standard.

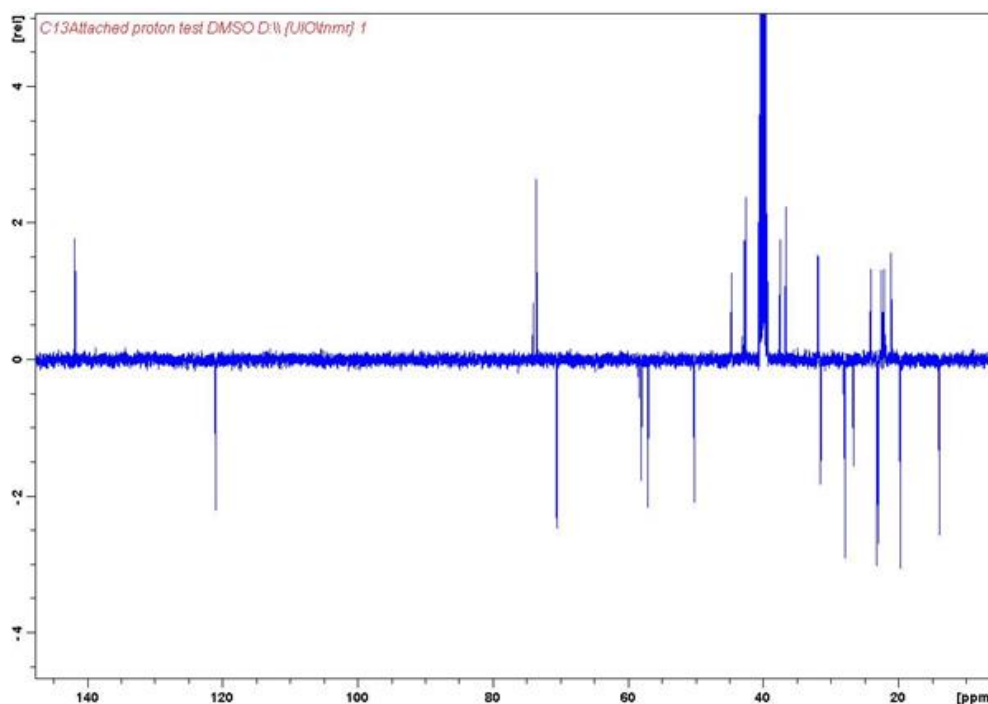


Figure 15 ^{13}C NMR spectra of $20\alpha\text{-OHC}$ standard. The molecule contains 27 carbon atoms and should give rise to 27 resonances if no impurity is present. More than 27 resonances were observed. CH_3 and CH are shown as negative peaks, while CH_2 and CH are shown as positive peaks. The multiplet at 40 ppm is residual 1H in the solvent DMSO_{d_6}

Another source of contaminants during analysis can be contaminations from reagents used during derivatization. In our hands, this was observed already in **Paper I**, as a late eluting peak during the wash out step to elute cholesterol from the column; this peak is visible in all standards and sample solutions and origins from the reagents (e.g. preparation of solution without oxysterol, only reagents give rise to the same peaks). These contaminants are shown in **Figure 16**, where contaminant from reagents elutes after 30 minutes. As these peaks have both the same parent m/z and fragmentation patterns as oxysterols, one can speculate if origins are from some cholesterol-similar molecules adsorbed to the cholesterol oxidase enzyme used in the sample preparation. These contaminants did not elute before the wash out step removing

cholesterol from the column, hence was not considered as a source of error during quantification.

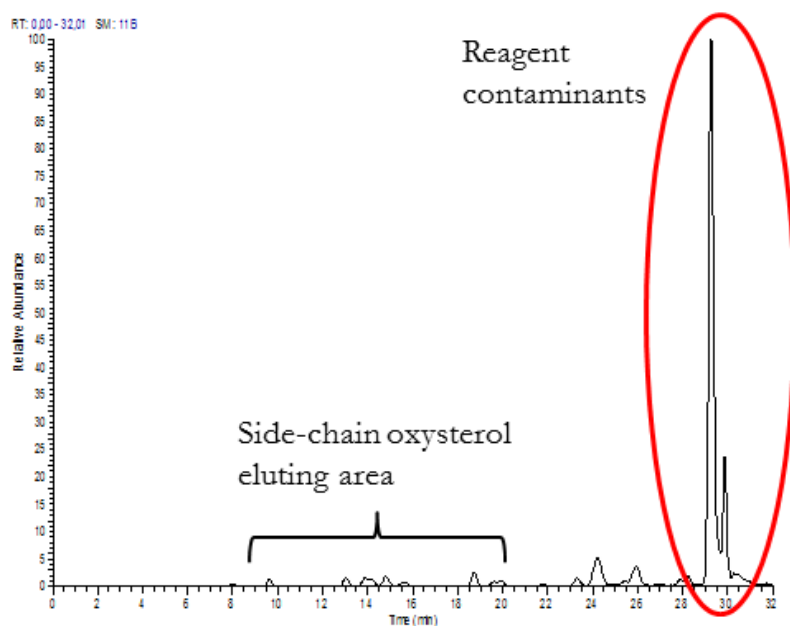


Figure 16 EIC (m/z 514.44 \rightarrow 445.36) of spiked cell lysate (50 000 cells spiked with 108 pM 22R-OHC, 24S-OHC, 25-OHC, 27-OHC and 22S-OHC) analysed on the AFFL-SPE-cap LC-MS/MS platform as described in paper IV. Contaminants from reagents elutes at 30 minutes, while side-chain oxysterols elutes at 9-20 minutes.

3.1.8 Separation of isomers

A separation is needed for determination of the oxysterol isomers, as these will have the same m/z and fragmentation pattern with ESI-MS detection.

In **Paper I**, an isocratic elution using ACN as organic solvent was used and showed good separation of the oxysterol standards available in our laboratory at that time. However, in the method development for **Paper II**, which includes more oxysterol standards, partial co-elution of 24S-OHC and 27-OHC was found with these elution conditions. The use of MeOH as organic solvent changed the selectivity on the ACE C₁₈ column providing separation of the two isomers. However, co-elution of the 27-OHC and 20 α -OHC isomers was observed when MeOH was used as organic solvent. Hence, samples should be analysed with both separation conditions to reveal the oxysterols present. 20 α -OHC has not been identified in concentrations above

detection limits in any samples analysed using ACN as organic solvent, questioning the reported endogenous presence of this analyte [21, 90, 91]. Efforts to separate 27-OHC and 20 α -OHC with combinations of ACN and MeOH as organic solvent has not been successful (data not published), as either co-elution of 27-OHC and 20 α -OHC or co-elution of other oxysterols have been observed. 22S-OHC, which was identified in samples in **Paper I**, is probably wrongly identified, as this oxysterol is not supposed to exist endogenously [42]. 22S-OHC was included in the study as it showed Hh activation [46, 55]. 22S-OHC was not identified in any samples using MeOH as organic solvent (**Paper II**, **Paper IV** and **Paper SIV**).

3.1.9 Concluding remarks on the developed methods

Using the AFFL-SPE system, robust platforms for on-line sample clean up and determinations of oxysterols were developed. The best performing platform regarding both chromatography and operation was the cap LC platform (column ID 0.3 mm). MDs (23-54 fg) and LODs (5-10 pg/mL) for the nano LC and cap LC platform were comparable or better than other published method (**Table 3**), and the methods are suitable for small sample analysis.

To further exploit the nano LC platform for oxysterol determination in samples of limited size some changes should be made. Firstly, different strategies for sample preparation should be explored. Even though the method used in **Paper II** is an efficient “in one vial” approach, the dilution of the sample is still large. Efforts to reduce final sample volume should be made. Reduced final sample volume would give a more concentrated sample; hence even a smaller sample starting size could be used. This might be of importance when analysing limited exosome samples. The nano LC method could also be used with a larger sample to explore low abundant oxysterols, not above detection limits in sample size used today.

For routine analyses, the cap LC platform was well performing and sensitive enough to detect oxysterols in limited exosome samples (See below). Hence

this should be the method of choice for larger sample sets. However, other stationary phases should be explored to separate 20 α -OHC from 27-OHC. Additionally, even though the method is faster than the nano LC method, the method is still long compared to routine methods for clinical laboratories e.g. by using ultra high performance LC (UHPLC). Potential timesaving steps could be explored, e.g. the use of core shell particles to gain faster and more efficient separation [17].

3.2 Determination of native oxysterols by nano LC

In **Paper III** we wanted to explore the possibilities to use nano LC to determine oxysterols without derivatization. In **Paper I**, **Paper II** and **Paper IV**, Girard T reagent was used to incorporate charged nitrogen into the analytes, enhancing the ionization of the analytes with ESI-MS. In general, the idea in **Paper III** was to examine if it was possible to utilize the high sensitivity gained with less radial dilution by nano LC to achieve acceptable detection limits of native oxysterols. In this way, derivatization of the analyte could be avoided, thus decreasing sample preparation time and avoiding dilution of the sample.

This work was inspired by the method of McDonald et al [44], who used formation of adducts to increase MS sensitivity of oxysterols. Unfortunately, with the MS instruments available in our laboratory, no stable adduct ions was created in positive mode using neither nano ESI nor ordinary ESI. In negative mode, oxysterol-acetate adducts ($[M+COO]^-$) was observed, but the ion signal was not stable between days.

In positive mode, loss of one or two water molecules was observed $[M+H-H_2O]^+$ and $[M+H-2H_2O]^+$. Such loss has previously been observed by others with APCI [49] and APPI [79]. Different solvents were examined to stabilize these ions, and a mixture of 2.5 mM ammonium formate (AF) and 0.25 % FA in either MeOH or 2-propanol (IPA) gave stable signal intensities between days. All solvents examined are shown in **Table 4**. Nonetheless, in MS/MS mode extensive fragmentation to low mass range ($m/z < 200$ Da) was

observed. This was found both by using a Quantiva™ tripleQ MS with collision induced dissociation (CID) fragmentation or Q Exactive™ Orbitrap MS with higher-energy collisional dissociation (HCD) cell. An example is shown using the Q Exactive™ MS in **Figure 17**. While the Girard T derivatized oxysterols give specific fragments with the loss of 59 Da, native oxysterols give extensive fragmentation in the low mass range, making it difficult to use MS/MS for identification and quantification.

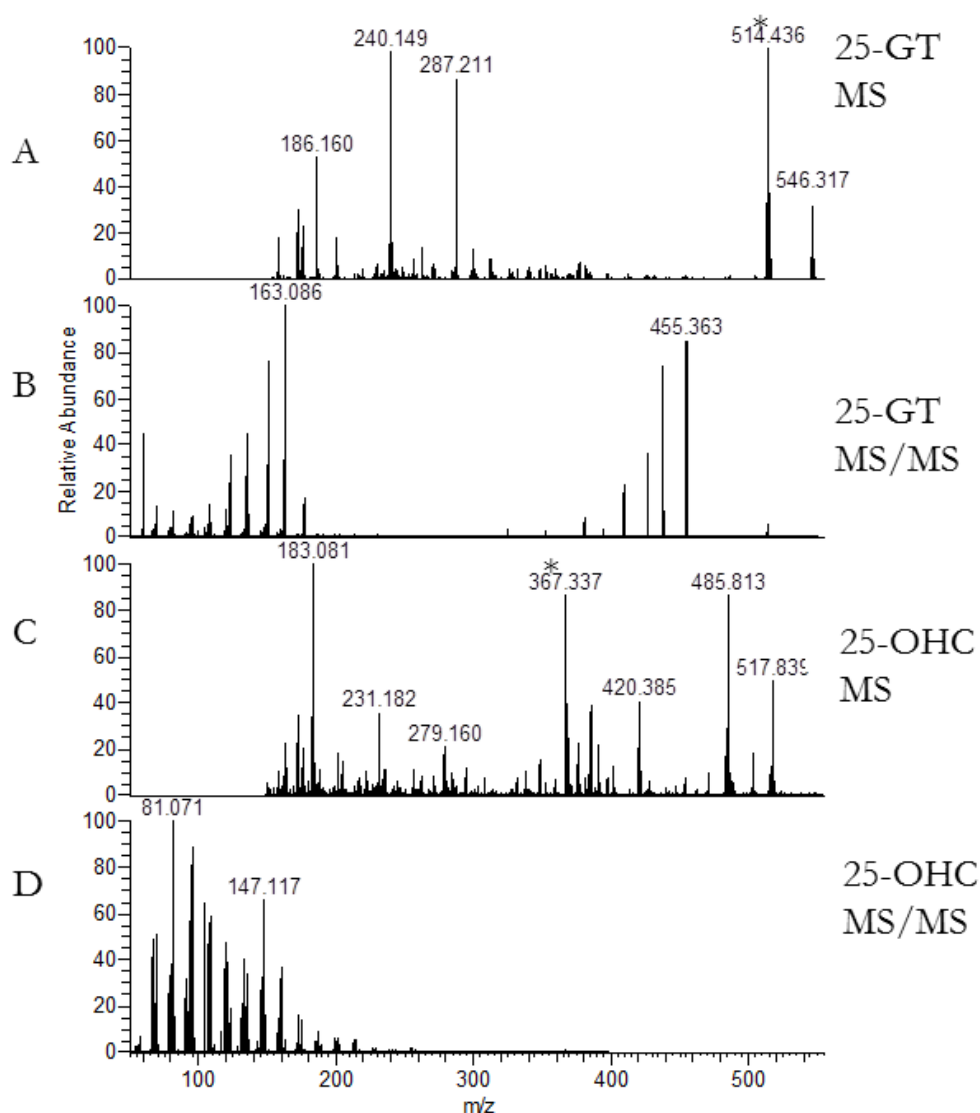


Figure 17 A) MS spectrum (m/z 200-550) of 1 $\mu\text{g/mL}$ Girard T derivate of 25-OHC dissolved in 0.1 % FA in MeOH. B) MS/MS spectrum of m/z 514.44 $[\text{M}]^+$. C) MS spectrum (m/z 200-550) of 1 $\mu\text{g/mL}$ 25-OHC in 0.25 % FA, 2.5 mM ammonium formate in MeOH. D) MS/MS spectrum of m/z 367.33 $[\text{M}+\text{H}-2\text{H}_2\text{O}]^+$ (Paper III).

Despite that no good and convincing MS/MS transition for native oxysterols was found, a nano LC-MS system for native oxysterols was further investigated. The high resolution Q Exactive™ Orbitrap MS instrument could be used as an alternative to MS/MS using SIM scan. Surprisingly, nano LC and underivatized oxysterols was not a good match. Extensive and persistent carry-over was observed and was localized to the narrow fused silica tubings (30 μm ID) needed to avoid extra column band broadening. This is shown in **Figure 18 (Paper III)** on a simplified system, consisting only of a pump, manual injector, spray needle (stainless steel) and MS, all connected with fused silica tubing. Repeatable, 10 injections of 25-OHC followed by 10 injections of mobile phase showed the same signal intensities in mobile phase as the analyte solution, implying that the origin of carry-over was the fused silica tubing. When increasing the ID of the fused silica (e.g. 75 μm), these effects were not observed (**Paper IV**).

To remove carry-over effects, the fused silica tubing was silanized with 5 % chlorotrimethylsilane. This will replace the silanol group on the surface of fused silica with methyl groups, hence possibly reduce adsorption of the oxysterols and remove carry-over effects. This attempt was successful for the simplified system (**Figure 18**), however when implementing it in a full scale nano LC system (without AFFL-SPE) the carry-over effects were still present. Another source of carry-over can be the column; however, the carry-over effects were not observed when native oxysterols were chromatographed in a larger scale system using the same ACE column material (column ID 1 mm, connected with 75 μm ID fused silica). This implies that the narrow tubing (ID < 30 μm) promotes self-adsorption of native oxysterols, possible due to the larger surface to volume ratio in narrow tubings compared to larger ID tubings.

These findings might have implications for other nano LC methods for biological samples with little sample preparation, as oxysterols and/or other steroids/lipids from the sample might adsorb to the fused silica surface and create extra column pseudo-stationary phases affecting the analysis.

Table 4 Solvents used for MS and LC investigations for native oxysterols (Paper III)

	Solvent direct infusion	Loading mobile phase	Eluting mobile phase	Results/comments
MS investigations	10 mM ammonium acetate in MeOH, IPA or ACN	-	-	No stable adducts in positive MS mode. No stable adducts from day to day in negative MS mode.
	10 mM acetic acid in MeOH	-	-	No stable adducts in positive MS mode. No stable Nano spray in negative mode.
	0.1-0.25 % FA in MeOH or IPA	-	-	Loss of one or two water molecules. Large variation in signal intensity.
	2.5 mM AF in MeOH or IPA	-	-	Loss of one or two water molecules. Large variation in signal intensity.
	AF/FA in MeOH or IPA	-	-	Loss of one or two water molecules. Stabile signal intensities
	10 mM AF in 70 % ACN	-	-	No signal.
LC investigations	-	0.1 % FA in H ₂ O	0.1 % FA in MeOH or IPA	Oxysterols trapped, large carry-over.
	-	0.1 % FA in MeOH (< 10 %)	0.1 % FA in MeOH or IPA	Oxysterols trapped, large carry-over.
	-	0.1 % FA in MeOH (> 10 %)	0.1 % FA in MeOH or IPA	No retention, carry-over not examined.
	-	0.1 % FA in EtOH (> 5 %)	0.1 % FA in EtOH	No retention, carry-over not examined.
	-	AF/FA in H ₂ O	AF/FA in MeOH or IPA	Oxysterols trapped, large carry-over.
	-	AF/FA in MeOH	AF/FA in MeOH or IPA	Oxysterols trapped, large carry-over.

		($< 10\%$)		
-	AF/FA in MeOH	AF/FA in MeOH or IPA	in	No retention, carry-over not examined.
		($> 10\%$)		
-	-	0.1 % FA in MeOH		Solubility issues, poor peak shape ($< 65\%$)
-	-	0.1 % FA in MeOH		Large carry-over. ($>65\%$)
-	-	0.1 % FA in IPA		Large carry-over.
-	-	AF/FA in MeOH	in	Large carry-over.
-	-	AF/FA in IPA	in	Large carry-over.

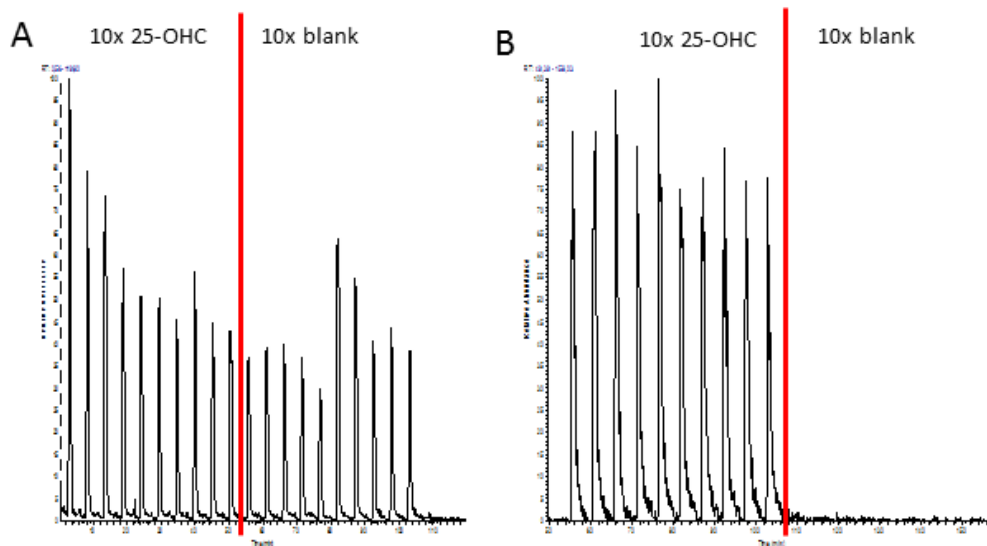


Figure 18 Signal of native oxysterols as a function of injection content and number on A) normal fused silica (30 μm ID) and B) silanized fused silica (30 μm ID)

3.3 Biological application of the methods

Methods developed in **Paper I** and **Paper II** have been used to determine oxysterols in cell samples in the context of the Hh signaling pathway. Oxysterols activates the Hh signaling pathway *in vitro*; however *in vivo* activation is still not understood [21, 48]. The methods developed in **Paper II** and **Paper IV** have been used to investigate oxysterols in the contexts of breast cancer. The nano LC platform was used to investigate 25-OHC and 27-OHC in ER+ breast cancer cells, and corresponding long-term estrogen deprived cell lines (**Paper SIV**), while the cap LC platform has been used to determine oxysterols in exosomes in connection to ER+ breast cancer (**Paper IV**). The exosome project is still ongoing in our lab, where a method for exosome isolation is under development.

3.3.1 Detection of Hedgehog active oxysterols

The method developed in **Paper I** was used to identify oxysterols in cancer cell line samples (HCT15 and HCT116), in addition to model cell lines often used for Hh signaling research (SHh-LII and 3T3). Also, a heterogeneous pancreatic cancer cell line (BxPC-3) sorted into cell subpopulations based on the epithelial to mesenchymal transition (EMT) was analysed. The method was sensitive enough to determine oxysterols in samples of 330 000 cells from cell subpopulation. The Vimentin positive cell subpopulation, which is more aggressive and invasive, showed a clear up-regulation of 24S-OHC compared to that in the less aggressive Vimentin negative cell subpopulation. As discussed above (3.1.8. Separation of isomers), 24S-OHC might have been wrongly quantified due to partial co-eluting with 27-OHC. 27-OHC was at the time in the study not included in the chromatographic method. The samples have not been re-analysed since the long term stability of the Girard T derivatives has not been examined.

When manipulating a Hh active cell line with a strong, recently developed Hh antagonist, MS-0022 [92], alteration of 24S-OHC levels was observed. When treated for 24 hours, the cells had a 3-fold decrease in 24S-OHC concentration.

The biological relevance and the role of the Hh signaling pathway in these variations are still unclear.

The results in **Paper I** are based on quantification of oxysterol per cell flask. Such quantification assumes that treatment of cells with antagonist does not affect cell growth. Ideally, the quantification should be normalized against either cell number (if the cells are of similar size, e.g. clones of the same cell line (**Paper II**)) or by protein concentration, to compare different cell lines (**Paper IV**). The experiments with MS0022 treatment were reproducible between assays and cell passaging.

The role of oxysterols in the Hh signaling pathway is still not fully understood. When using MS0022, 24S-OHC (and possible 27-OHC) was downregulated (**Paper I**), while using an Hh agonist, SAG, 27-OHC was upregulated (data not published). Nachtergaele et al have shown that oxysterols are allosteric activators of SMO [21] and that oxysterols bind to the extracellular cysteine rich domain of SMO [47], hence activating the Hh signaling pathway. Although manipulation of the pathway with e.g. MS0022 did affect the concentration of the oxysterols, the biological implication this might have regarding cancer still remains to be revealed.

3.3.2 Detection of oxysterols in exosomes

Exosomes are small (30 to 100 nm) extracellular vesicles released from all kinds of cells by exocytosis [93-96]. Previously thought to be of no special interest, later research has shown that these exosomes can carry metabolites and proteins and are heavily involved in intercellular communication [96, 97], including cancer metastasis [98, 99]. Cancer cells generate more of these exosomes [94, 97] and it is speculated that these exosomes are involved in organ specific metastasis of the cancer [97-99]. Also, the Hh protein activating the Hh signaling pathway is delivered by extracellular vesicles [53]. Exosomes can contain high amount of lipids [100] and might play an important role in diagnosis and characterization of a tumor, as biomarkers and signal molecules

most probably are enriched [101] and present in a simpler matrix compared to blood and whole cell lysate.

The difficulty with exosome analysis is the isolation of the pure exosomes samples without contamination of other larger/smaller extracellular vesicles [102, 103]. The most used technique for isolation is ultracentrifugation, but commercial kits are also available [97]. Common for these techniques is that the exosome yield is mostly low; hence a sensitive analytical method is needed to further analyse these samples.

With the AFFL-SPE-cap LC-MS platform we were able to identify oxysterols in commercial available exosomes from an ER+ breast cancer, ER- breast cancer, human embryonic kidney cell line and human pooled serum. Compared to the cell samples, 27-OHC was highly enriched in the exosomes from ER+ breast cancer cell line, while 24S-OHC and 25-OHC did not follow the same trend (**Figure 19**). Also, this profile did not match the profile in the cell lines, where 27-OHC was found to be highly concentrated in ER- breast cancer cell line, and only modestly expressed in ER+ cancer cell line compared to other cancer (Pancreatic and colon) or human embryonic kidney cell line.

27-OHC has been found to be closely connected with breast cancer, especially ER+ breast cancer [22, 23, 40]. Nonetheless, the measurements have often been performed by measuring e.g. CYP27A1 or CYP7B1, instead of the metabolite itself [22]. mRNA, protein and active metabolite concentration does not always correlate [71-73](**Paper II**), and in **Paper IV**, this was shown when comparing the concentration of 27-OHC with protein concentration and mRNA expression of CYP27A1 in cancer cells (**Figure 20**). While 27-OHC had highest concentration in MDA-MB-231, an ER- breast cancer cell line, the CYP27A1 concentration in all cell lines, measured by WB, appeared similar. WB quantification for these cell samples was, however, difficult, probably due to the modest sensitivity of the assay.

A close examination of the WB reveals two bands for the MDA-MB-231 cell sample in the correct mass area on the WB membrane (**Figure 20B**). This

might be due to some isomers of CYP27A1 or a contamination that binds the antibody used for WB. Further proteomic LC-MS/MS investigations should be made to identify the bands correctly, and this demonstrates the importance of having good analytical tools available in diagnostics and research.

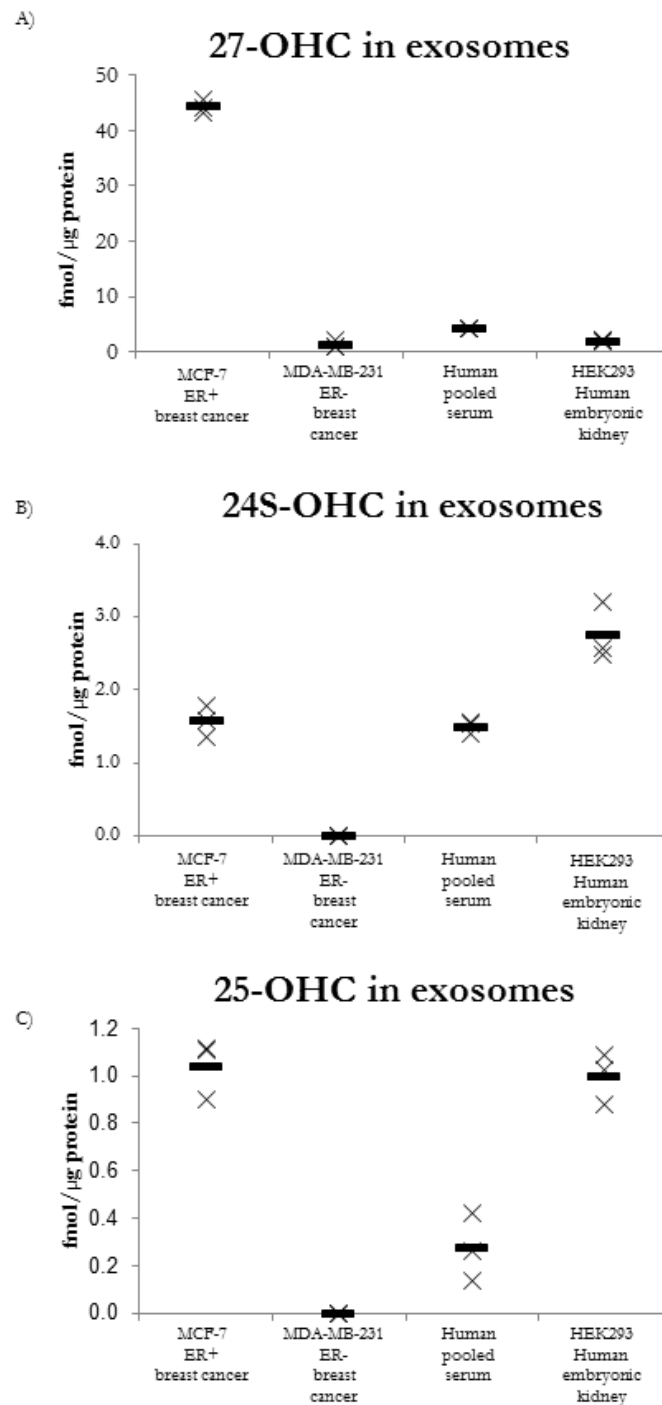


Figure 19 Concentration of A) 27-OHC, B) 24S-OHC and C) 25-OHC in exosomes from breast cancer, human pooled serum and human embryonic kidney cells (Paper IV).

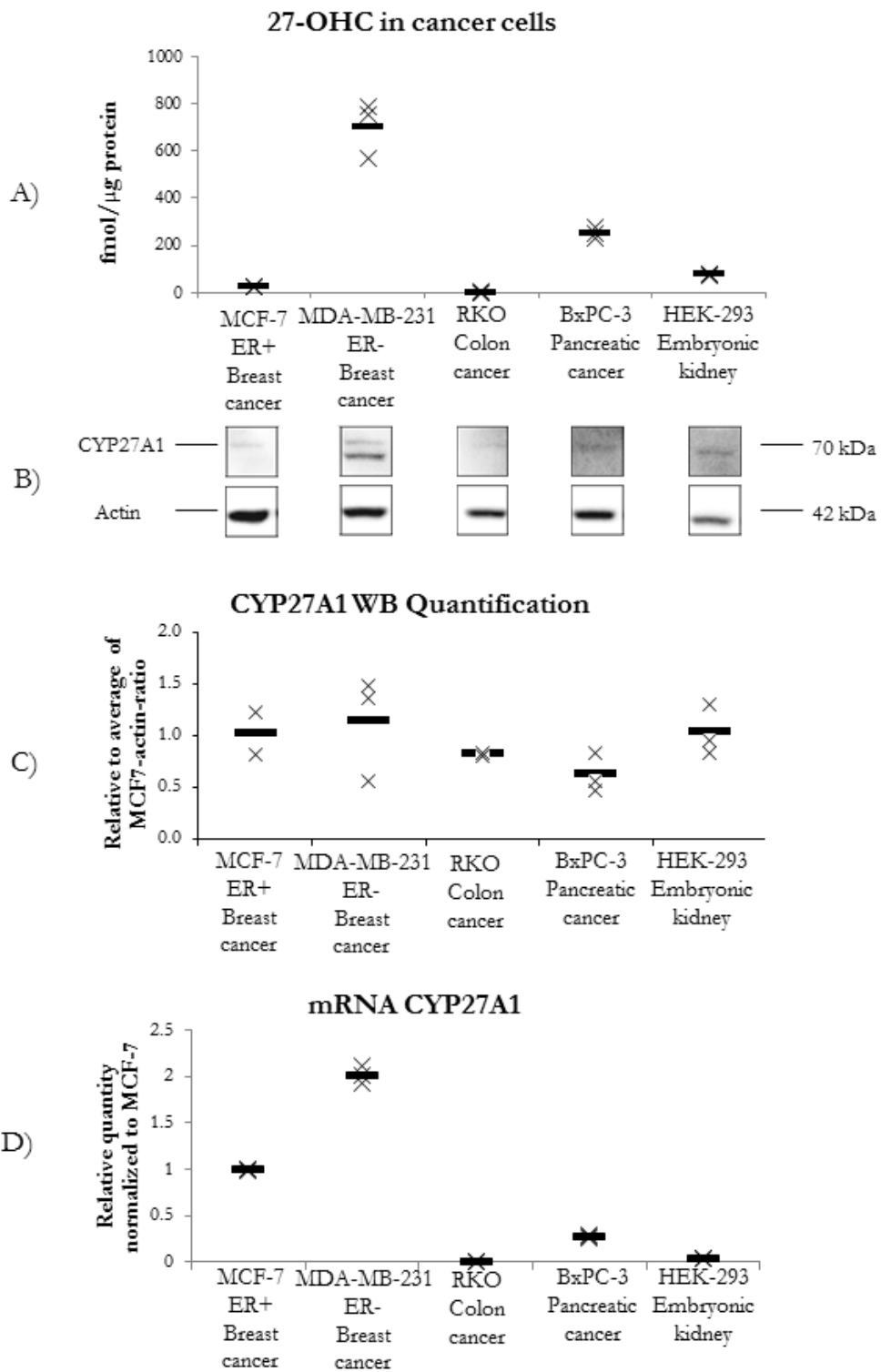


Figure 20 Comparison of metabolite (27-OHC), protein/enzyme and transcription (mRNA) concentration in cancer cell lines (Paper IV).

3.3.3 Concluding remarks on oxysterols and cancer

The application of the published methods in this thesis shows the potential to further explore oxysterols in relation to cancer. Regarding breast cancer, the nano LC method was used to analyse ER+ breast cancer cell lines together with corresponding long-term estrogen deprived cell lines (LTED) (**Paper SIV**). All the LTED had upregulated cholesterol biosynthesis pathway, implying that cholesterol and oxysterols have an effect on aromatase inhibitors, the most common way to treat ER+ breast cancer. In this study, 25-OHC and 27-OHC showed SERM activity and could rescue proliferation in the LTED cells.

27-OHC has been closely connected with breast cancer and in particular ER+ breast cancer [22, 23, 40]. However, complete function and role of oxysterols in cancer in general and in particular breast cancer are not completely explored. By employing the methods developed in this thesis on small cell subpopulation and exosomes, oxysterols and their potential role in cancer can be investigated.

CYP27A1 is responsible for converting cholesterol into 27-OHC, and this enzyme is primary found in macrophage cells. 27-OHC can act as a SERM for both ER and LXR. Tumor associated macrophages [104] might play an important role in proliferation by feeding tumors with 27-OHC [22] and metastasis by activation of LXR with 27-OHC [22], perhaps by exosomes. The cancer exosomes seems to be organ specific [99], e.g. MDA-MB-231 exosomes metastasis to lungs while BxPC-3 exosomes metastasis to liver [105]. Hence determination of oxysterol in exosomes might have a diagnostic role in cancer in general and in particular breast cancer.

4 Conclusions and further perspective

This thesis presents methods for determination of cancer related side-chain hydroxylated oxysterols. By using the AFFL-SPE system, robust on-line sample clean-up can be performed. In combination with nano LC and cap LC, low detection limits are achieved, making detection of oxysterols in cell subpopulation possible. Of the methods, the cap LC platform performed best regarding time, chromatographic performance and LOD. To better exploit the nano LC system, a higher injection volume or another strategy for derivatization of oxysterols should be examined. Also, user-friendly lower dead volume equipment must be further developed to fully utilize the entire performance of the nano LC system [17].

To determine oxysterols in limited samples using nano LC a derivatization is needed to avoid extreme and persistent carry-over, mainly caused by self-adsorption of oxysterols to fused silica tubing. Other materials for tubings in nano LC could be explored. This might have implication for analysing biological samples with nano LC, as oxysterols (and other sterols) might adsorb to tubings creating carry-over effects if not removed before analysis.

Oxysterols are connected to cancer through ER, LXR and Hh signaling pathway. Especially 27-OHC, a potent ER and LXR activator is closely connected to breast cancer. This isomer was identified in higher concentrations in exosomes from ER+ breast cancer cells compared to cells and exosomes from ER- breast cancer cells.

The findings that 27-OHC is involved in both ER+ α and LXR activation makes the CYP27A1 enzyme a potential target for cancer drug development. However, the drug must be specific; both in targeting CYP27A1 and in location, as inhibiting CYP27A1 in e.g. liver would not prevent cancer but bile acid production. One way of targeting CYP27A1 in tumor associated macrophages could be by using nanoparticles loaded with CYP27A1 inhibitors. This approach has already been successfully used in targeting other macrophage specific diseases, such as tuberculosis [106].

With the methods developed and presented in this thesis, oxysterols are determined in cancer cell subpopulations and cancer derived exosomes are possible. A difference in oxysterol profile from cancer cells and cancer derived exosomes are shown in ER+ breast cancer cell lines, connecting 27-OHC in exosomes to ER+ breast cancer. This link needs to be further investigated. The connection between oxysterols and Hh signaling pathway is still unclear.

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