Determination of CYP27a1 in biological samples using nano liquid chromatography mass spectrometry

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May 2016

Year: 2016

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Preface

The work of this master thesis was performed at the Department of Chemistry in the group Bioanalytical Chemistry and at the Rikshospitalet in the group Unit for Cell Signaling. My supervisors were PhD candidate Tore Vehus, PhD candidate Hanne Røberg-Larsen, Professor Stefan Krauss, Professor Elsa Lundanes and Associate Professor Steven R.H. Wilson.

First, i would like to thank all of my supervisors for their guidance and motivation throughout the two years of my master thesis, and I would like to express my gratitude to all of my co-workers in the Unit for Cell Signaling for their patience and help with my attempts to work with molecular biology, and for including me in their group. Also I would like to thank the entire Bioanalytical Chemistry group for providing a great social environment.

Oslo, Norway, May 2016

Kristina Erikstad Sæterdal

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1 Abbreviations and definitions

1.1 Abbreviations

ABC	Ammonium bicarbonate
ACN	Acetonitrile
AFFL	Automated filtration/filter backflush
AGC	Automatic gain control
AMP	Ampicillin
ATCC	American Type Culture Collection
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CA	Citric acid extract
Chir-IP	Chromatin immuno precipitation
CRM	Charge residue model
CYP	Cytochrome P450
DMEM	Dulbeco's modified eagle medium
dMS/MS	Data dependent tandem mass spectrometry
DMSO	Dimethyl sulfoxide
DQ	Direct-Q®
DTT	Dithiothreitol

E. coli	Escherichia coli
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's minimum essential medium
ESI	Electrospray ionization
ESP	Enhanced signature peptide
FA	Formic acid
FBS	Fetal bovine serum
FFT	Fast Fourier transformation
GC	Gas chromatography
GE	Gel electrophoresis
H	Human embryonic kidney 293 lysate
HEK293	Human embryonic kidney 293
Hh	Hedgehog
HPLC	High pressure liquid chromatography
HRP	Horseradish peroxidase
i.d.	Inner diameter
IAM	Iodoacetoamide
IEM	Ion evaporation model

Ig	Immunoglobin
IP	Immunoprecipitation
IT	Injection time
ITS	Insulin-transferring-selenium
KI	Department of Chemistry
LB	Lysogny broth
LC	Liquid chromatography
LC-MS	Liquid chromatography mass spectrometry
M	MDA-MB-231 lysate
m/z	Mass/charge
MALDI	Matrix-assisted laser desorption
MCF7	Michigan Cancer Foundation 7
MIK	Department of Microbiology
MP	Mobile phase
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NCE	Normalized collision energy
NP-40	Nonyl phenoxypolyethoxylethanol
OHC	Hydroxycholesterol
OHP	Overhead projector
ON	Overnight supernatant

OUS	Oslo university hospital
P/S	Penicillin/streptomycin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline with 0.1 % Tween® 20
PRIDE	Proteomics identifications database
PRM	Parallel reaction monitoring
PS-DVB	Polystyrene divinylbenzene
PVDF	Polyvinylidene fluoride
RP	Reversed phase
RPMI	Roswell Park Memorial Institute
RSD	Relative standard deviation
S	Standard deviation
S/N	Signal to noise
SDS	Sodium dodecyl sulfate
SERM	Selective estrogen receptor modulator
Sigma	Sigma Aldrich
SILAC	Stable isotope labelling with amino acids in cell culture
SP	Stationary phase
SPE	Solid phase extraction
TBS-T	Tris buffered saline tween

TFA	Trifluoroacetic acid
Thermo	Thermo Scientific
tMSMS	Targeted tandem mass spectrometry
Tr	Human embryonic kidney 293 transfected lysate
W1	Wash no. 1 supernatant
W3	Wash no. 3 supernatant
WB	Western blot

1.2 Definitions

Full MS	The MS scan the precursor ions, and no fragments are formed
Precursor ion	The entire ion that entered the mass analyser. Form fragment ions when fragmented
Fragment ion	The resultant ion after fragmentation of the precursor ion in the collision cell
Tandem MS (MS/MS)	The MS fragments the precursor ion, and scan the fragment ions
Data dependent MS/MS	The MS fragments precursor ions meeting a set of demands (e.g. intensity)
Cell lysis	Disruption of the cell membrane
Cell	The basic biological unit of all living organisms
Cytochrome P450	A group of enzymes
Mycoplasma	A bacteria that is a contaminant in laboratory cell work
Confluence	Describes the proportion of growing area the cells cover
Injection time	The time the Orbitrap collects ions before scanning
Automatic gain control	The amount of ions the Orbitrap collect before scanning
MS – scheduling	Defining a retention time window for a precursor ion

Parallel reaction monitoring	Defining a list of precursor ions to monitor when applying an Orbitrap MS (tMS/MS)
Resolution	The ability of a mass spectrometer to separate ions by *m/z*. Is calculated by dividing the observed *m/z* value by the smallest difference in *m/z* for two ions that can be separated
Retention time	The time a compound uses from it is injected on the column and until it reaches the detector
Cell culturing	Growing cells in an artificial environment in a laboratory

2 Abstract

The cytochrome P450 enzyme, CYP27a1, converts cholesterol into 27-hydroxycholesterol, and both the metabolite and the enzyme are associated with breast cancer, promoting proliferation and metastasis. CYP27A1 is enriched in cancer tissues, and the amount of CYP27a1 correlates to tumor grade. Hence, CYP27a1 is a potential biomarker, and a method for determination and quantification is needed. Antibody assays shows limited performance, and therefore the goal of this work was to develop a nano liquid chromatography mass spectrometry (LC-MS) method for determination of CYP27a1. A sample preparation workflow for CYP27a1 in cell samples was investigated. CYP27a1 was identified by nano LC-MS in MDA-MB-231 breast cancer cells by fractionating cell lysates using gel electrophoresis. Enrichment of CYP27a1 in cell samples using immunoprecipitation, an antibody based method for protein extraction, did not provide adequate identification. However the former method works when looking at other proteins, and this confirms that there are limitations in using antibody based methods for determination and quantification of CYP27a1.

3 Introduction

3.1 Background

3.1.1 Cancer

Breast cancer – proliferation and metastasis

Breast cancer is one of the cancer types women are most exposed to; in 2014, 3324 women were diagnosed with breast cancer in Norway [1, 2] (***Textbox 1***), and 1.7 million were diagnosed worldwide in 2012. Today, detection and diagnosis of breast cancer are based on image scanning by mammography. However, mammography is a method prone to false positives [3, 4]. The majority of breast cancers are estrogen receptor positive, meaning that the cancer cells have receptors sensitive to the hormone estrogen. When hormones bind to the receptors on the cell it promotes proliferation. These receptors are selective to estrogen, but other endogenous ligands, such as oxysterol 27-hydroxycholesterol (27-OHC), are found to be selective estrogen receptor modulators (SERM) [5, 6]

Textbox 1 Breast cancer in Norway

Breast cancer in Norway

Breast cancer is the most abundant cancer type among women in Norway. It is a risk of 8.4 % to develop breast cancer before the age of 75 years for women (0.1 % for men). The relative survival rate is now up to 90 %, an increase from 70 % in 1979 [2]. Today, women are offered a regularly mammogram from the age of 55. In an evaluation of the mammogram program performed by The Research Council of Norwegian [7], they concluded that the program reduced the risk of death by breast cancer by 30 %. But the evaluation also reported an overdiagnosis estimate of 20 % [7].

3.1.2 Oxysterols

Oxysterols are cholesterols with an additional OH-group. The oxidation (addition of OH-group) is either non enzymatic (autoxidation [8, 9]) or catalysed by the cytochrome P450-family (CYP enzymes). Where the OH-group is located on the cholesterol branch depends on the CYP catalysing the reaction, and the OH-location is important for the metabolic activity [10-12]. Oxysterols are closely connected to the Hedgehog signalling pathway (***Textbox 2***)

which is central in numerous cancers [13-16]. 27-OHC and its enzyme are also closely related to breast cancer and this has been the focus in this thesis.

27-Hydroxycholesterol (27-OHC) is an endogenous oxysterol with the OH-group located on the cholesterol side-chain (***Figure 1***). Its systematic name is 25R-26-Hydroxycholestrerol, but in medicine the name 27-OHC is the most used [17], and is the name to be used in this thesis. 27-OHC increases the risk of breast cancer [18, 19]. The metabolite promotes proliferation (growth) and metastasis (spreading) of breast cancer, by binding to the estrogen receptor or the liver X receptor, respectively, in breast cancer cells [18-20]. 27-OHC is therefore a potential biomarker for breast cancer and can be determined in cancer cells at femtomol/10 000 cells-levels, using an automated (LC-MS) method [21].

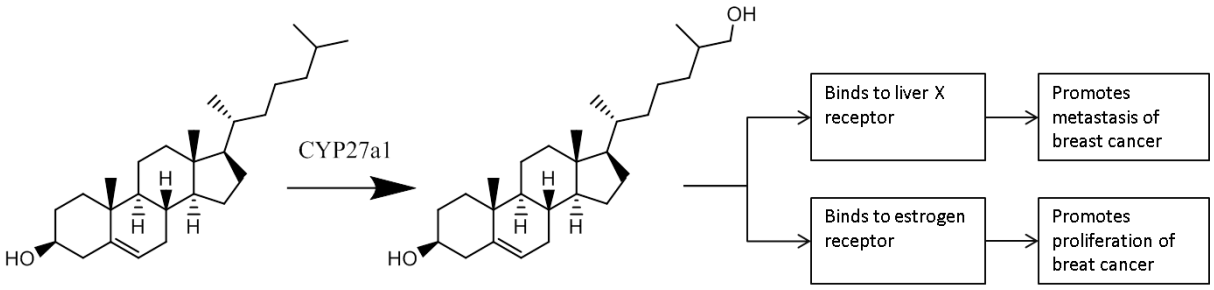


Figure 1 Formation of 27-hydrocholesterol. 27-hydroxycholesterol is formed by adding an OH-group to the side-chain of cholesterol. *The oxidation is either autoxidation or catalysed by CYP27a1. 27–OHC binds to the liver X receptor and to the estrogen receptor, promoting metastasis and proliferation of breast cancer, respectively.*

3.1.3 CYP27a1

The enzyme CYP27a1 (also called sterol 27-hydroxylase, see ***Table (AP) 4*** for protein sequence) [22] catalyses the oxidation of cholesterol to the oxysterol 27-OHC (and 3β-hydroxy-5-cholestenoic acid) [23, 24]. As 27-OHC increases the risk of breast cancer, CYP27a1 must also be addressed. In cancerous tissues (e.g. macrophages [18, 25]) the amount of CYP27a1 correlates to tumor grade [18], and interfering with the CYP27a1 conversion of cholesterol to 27-OHC, may be a way to treat or prevent breast cancer [18].

Hence, CYP27a1 can be an important biomarker for breast cancer and methods for measuring the enzyme are called for [20].

Textbox 1 The Hedgehog signaling pathway

Signaling Pathways – the Hedgehog signaling pathway

A signaling pathway is a part of the communication system of the body. During a cascade of protein activations, a signal is sent to the cell, which responds by proliferation. If a signaling pathway is exposed to mutations or other external influences, like metabolites, it could lead to inhibited cell growth (cancer) due to overexpression in the cell.

The Hedgehog (Hh) signaling pathway [26] is highly important in embryonic development and cell growth [27]. An irregulation in Hh signaling is associated with diseases and development of numerous cancers [13-16, 28]. Oxysterols are suggested to play a role in promoting cancer development by activating the Hh signaling pathway [13, 14]. The correlation between 27-hydroxycholesterol and CYP27a1 with Hh signaling has not been proven, but the preliminary goal of this thesis was to investigate it as preliminary research showed an regulation of 27-OHC in tumor initiating cancer cells [21].

3.2 Targeted proteomics

3.2.1 Targeted proteomics

How are proteins measured? The proteome [29-31] refers to the entire protein content, and proteomics [32, 33] is the large-scale study of proteins. In "targeted proteomics" [34, 35], the aim is to detect specific proteins in a sample, e.g. for diagnostic purposes.

Western blotting

Today the dominant methods for protein quantification are ELISA (enzyme-linked immunosorbent assay) [36] and western blot (WB) [37] which rely on antibodies (***Textbox 3***) for protein detection ("immunoassays").

In WB (***Figure 2***) proteins are electrophoretically separated [38] on a gel according to their size [39, 40] (see ***section 3.3.4).*** Following separation, an applied voltage is used to transfer the proteins from the gel and onto a membrane made of nitrocellulose or polyvinylidene fluoride (PVDF).

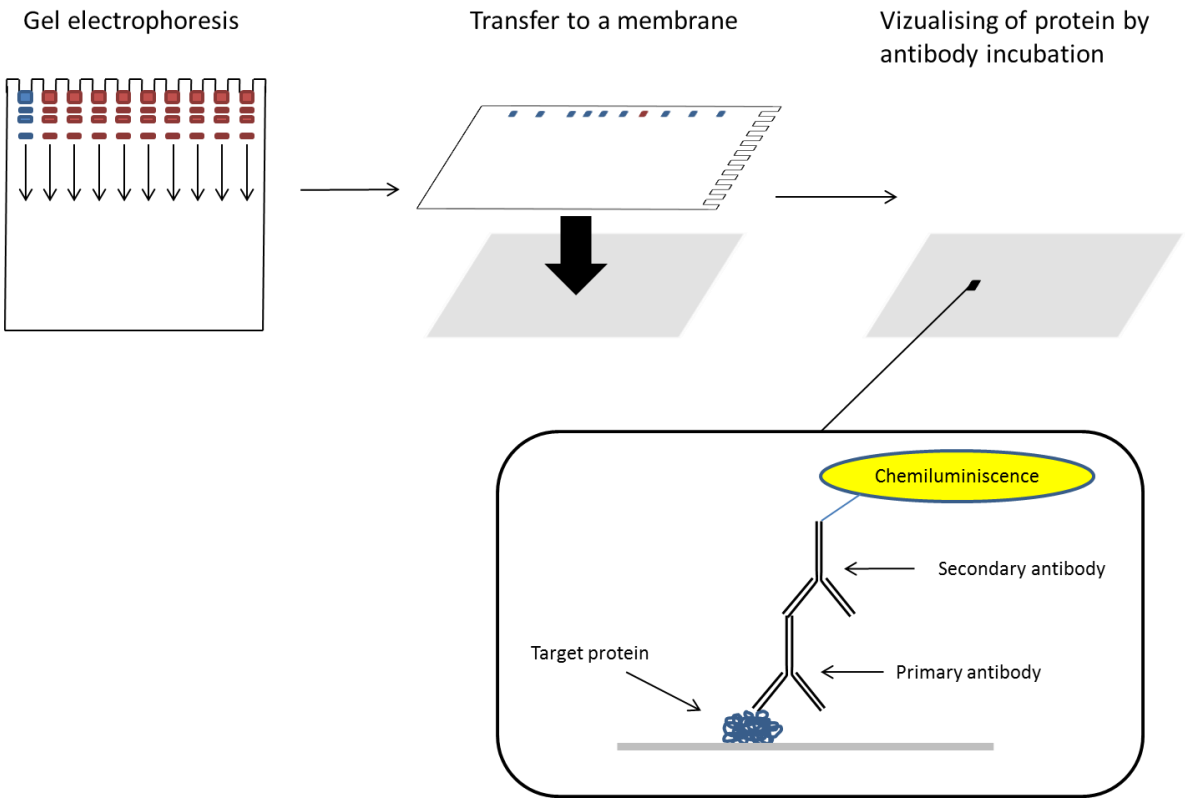


Figure 2 WB procedure. *During a WB procedure, proteins are electrophoretically separated on a gel, before they are transferred to a membrane. The membrane is subsequently exposed to primary, and secondary, antibody solutions, for detection of selected protein(s).*

After several steps in which the membrane is incubated, it is possible to visualize the protein, and the amount: During the first incubation the membrane is covered with a solution containing proteins to prevent nonspecific binding of the antibody on the membrane. This liquid can be a bovine serum albumin (BSA) solution or milk. A diluted antibody solution (a primary antibody that is specific for the target protein) is then added on the membrane, and it binds to the target protein. However, neither the protein nor the antibody exhibit features making it visible (e.g. luminescence), so the membrane is finally incubated in a secondary antibody solution. This antibody is specific towards the primary antibody, e.g. if a rabbit-antibody is used as primary antibody, the secondary antibody must be an anti-rabbit antibody, enabling it to bind to the primary antibody (***Textbox 3***). The addition of a labeled secondary antibody enables detection (e.g. labeling with horseradish peroxidase (HRP) enables detection by chemiluminescence [41]).

WB is dependent on the selectivity of an antibody for protein detection (***Figure 3***). Many proteins however, e.g. CYP27a1, do not have a specific antibody ensuring a selective detection. Unselective antibodies recognize other proteins in addition to the ones being targeted. In addition the sensitivity provided by antibodies is variable; some antibodies provide an outstanding sensitivity, but for the most of them, the sensitivity is poor. In the past years a "reproducibility crisis" [42] has evolved, referring to that many antibody based experiments are not reproducible due to the antibodies [42]. Therefor there is a need for more selective methods. By developing a targeted LC-MS method more selectivity can be achieved.

Figure 3 WB showing using a CYP27a1 antibody. *Several bands appear, but only the lower band is appearing at the correct mass for CYP27a1.*

Textbox 3 Antibodies

Antibodies

An antibody is a Y-shaped protein, also called immunoglobin (Ig) [43, 44]. Antibodies have the ability of specific bindings towards an antigen, e.g. a protein. An antibody is a key component in the human body as it binds to a specific bacterium, neutralizing it.

The Y shape is made up by two identical heavy chains (large) and two identical light chains (small), connected by disulfide bonds (***Figure A***). There are five different antibodies; IgA, IgD, IgE, IgE and IgM. IgG is the highest abundant antibody in humans, and is most used in immunobiology. Antibodies have different animal hosts based on where they are produced; mouse, rabbit, goat, rat, horse and sheep.

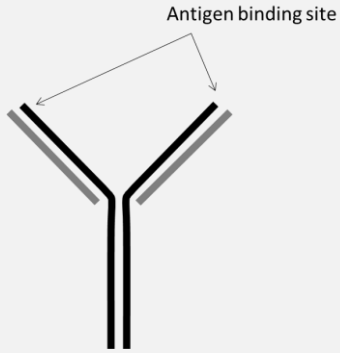


Figure A The construction of an antibody. *An antibody is built up by two equal heavy chains (black) and two equal light chains (grey), connected through disulfide bonds. The antibody regions where the light chains are located are the regions that are selective towards the antigen, e.g. a protein.*

In immunobased biology, like in WB, the selective bindings of antibodies are used for detection. The strength of the antigen-antibody binding differs based on the antibody in question. An antibody is said to be "good" when the bonding to its antigen is strong, which also prevent cross-reactions with other antigens, and hence ensures specific detection.

3.2.2 Mass spectrometry

One solution to the issue with selectivity in WB is to use a mass spectrometer (MS). With MS, ions are detected based on their mass/charge (*m/z)* value, and are visualized in a mass spectrum. Either the intact molecular ion (precursor ion) could be detected (MS-mode), or it could be fragmented into fragment ions (tandem-MS (MS/MS)). MS/MS can provide enhanced selectivity, as the MS/MS fragment ions provides additional information of the compounds identity.

Several mass spectrometers are applicable for the study of proteins (e.g. time of flight, triple quadrupoles and Orbitrap). The quadrupole Orbitrap instrument used in this work will be described below.

In the Orbitrap, ions are trapped in an orbit around an electrode, and the time-domain signals are converted into mass to charge spectra using a fast Fourier transformation (FFT) [45]. The Q Exactive [46], which is used in this thesis, is equipped with a quadrupole situated before the Orbitrap to achieve mass filtration of the precursor ions. A quadrupole consists of four parallel cylindrical rods with applied voltage. These rods prevent ions other than those with a chosen *m/z*-ratio to pass. By varying the voltage applied, the operator can scan for a range of *m/z*-values. In full scan MS all ions enter the Orbitrap to be scanned, while in MS/MS mode ions are fragmented in the collision cell prior to the Orbitrap. The Q Exactive ensures high resolution (up to 140 000) and high mass accuracy [46].

Parallel reaction monitoring

Parallel reaction monitoring (PRM) [35, 47] is an Orbitrap variant of targeted MS/MS (tMS/MS). A schematic overview of the PRM in a Q Exactive is illustrated in ***Figure 4.*** The MS is told which ions are allowed to pass the quadrupole and subsequently fragmented in the collision cell. All fragments of the corresponding ion are sent to the Orbitrap to be scanned.

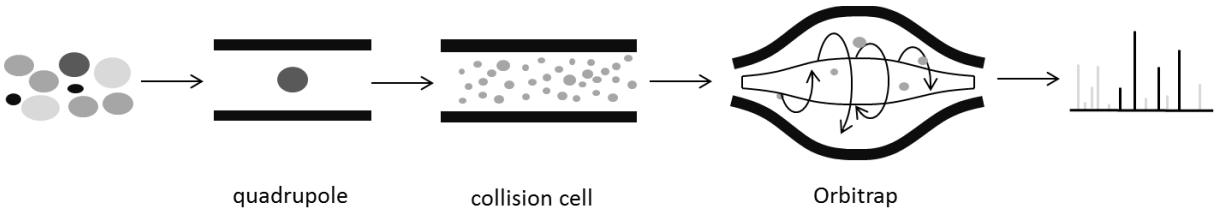


Figure 4 Parallel reaction monitoring. *Ions enter the quadrupole, and the ions with selected m/z-values are allowed to pass and enter the collision cell, where they are fragmented. Subsequently ions enter the Orbitrap to be detected. The figure is adapted from* ***[47]***

Digestion

Peptides (bottom-up method) (***Figure 5***) provides higher sensitivity and less complex spectra than intact proteins (top-down method [48]) [49]. The decrease in spectra complexity is because peptides contain a limited sequence; the number of different charges and possible modifications are less [49]. Identifications of proteins are ensured through fragmentation spectra of their corresponding peptides.

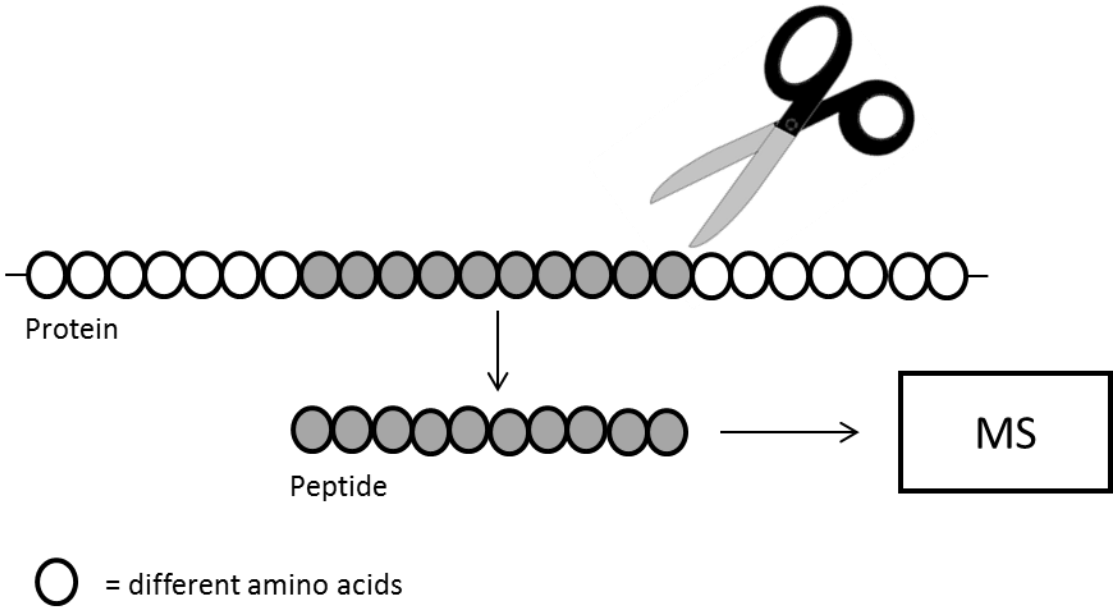
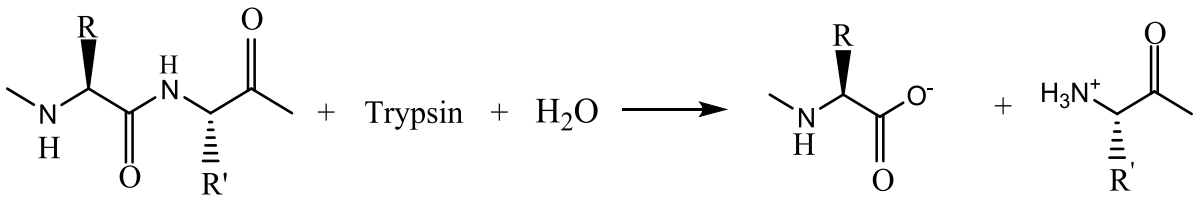


Figure 5 The bottom-up methods used in proteomics. *Proteins are cleaved into peptides before LC-MC determination.*

Trypsin is a protease that cleaves proteins at the carboxyl side of the amino acids lysine (K) and arginine (R), except when they are followed by proline (P) [50]. The cleavage reaction (hydrolysis) is illustrated in ***Figure 6.*** Trypsin is commonly used in bottom-up proteomics [49] due to its selectivity [50] and appropriate size of the resulting peptides.



R= arginine or lysine

Figure 6 Hydrolysis of proteins by trypsin. *When a protein is exposed to trypsin, trypsin cleaves the protein at the carboxyl side of the amino acids lysine and arginine by hydrolysis.*

Peptide fragmentation

Peptide fragmentation in the MS collision cell occurs on the peptide bond and fragment ions denoted y-ions and b-ions are produced (***Figure 7***). The y-ions are numbered counting from the carboxyl residue, while b-ions are named counting from the amino residue.

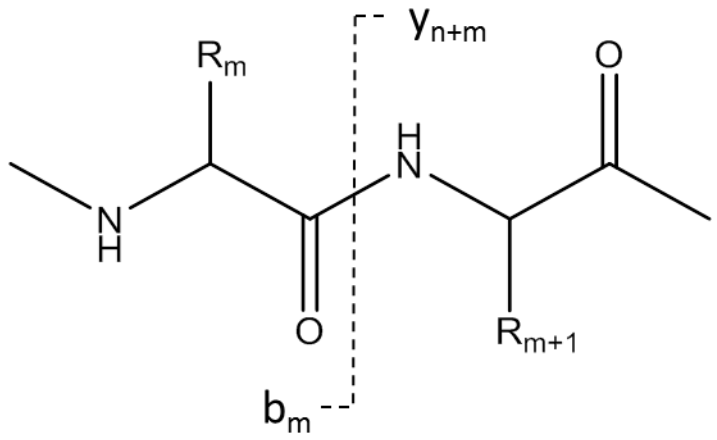


Figure 7 Peptide-fragmentation in MS. *The peptide is cleaved at the peptide bond, producing y and b fragment ions.*

Choosing appropriate signature peptides

To obtain a secure identification of a parent protein in bottom-up targeted proteomics, the peptide must be unique, meaning that no other proteins can produce the peptides, hereafter called signature peptides. Furthermore, the signature peptides cannot be subject to uncontrolled reactions/modifications which affect the reproducibility. Peptides containing cysteine (C, oxidation and carbamidomethylation), methionine (M, oxidation) [51] and tryptophan (W, oxidation) [52] are avoided; see ***Figure 8***, ***Figure 9*** and ***Figure 10***, respectively, for reactions. Peptides containing sequences often missed in cleavages (e.g. lysine-lysine and argenine-argenine) may also be excluded [51]. An overview of all amino acids with abbreviations is found in ***Table (AP) 5*** in appendix.

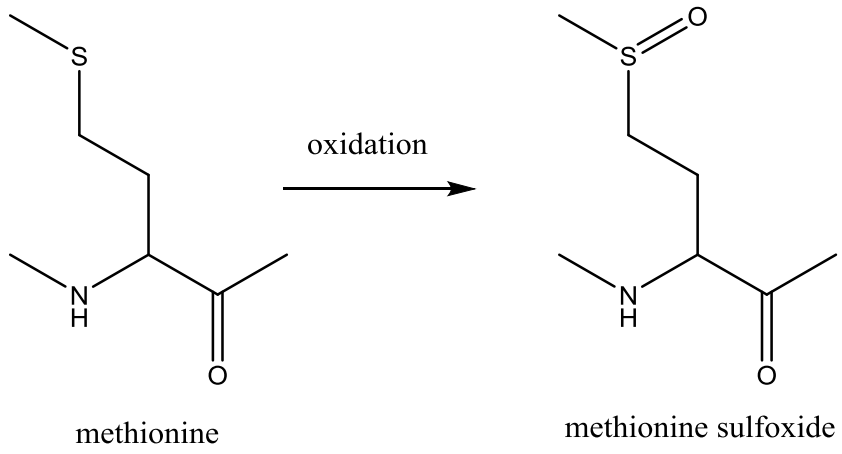


Figure 8 The oxidation of methionine to methionine sulfoxide.

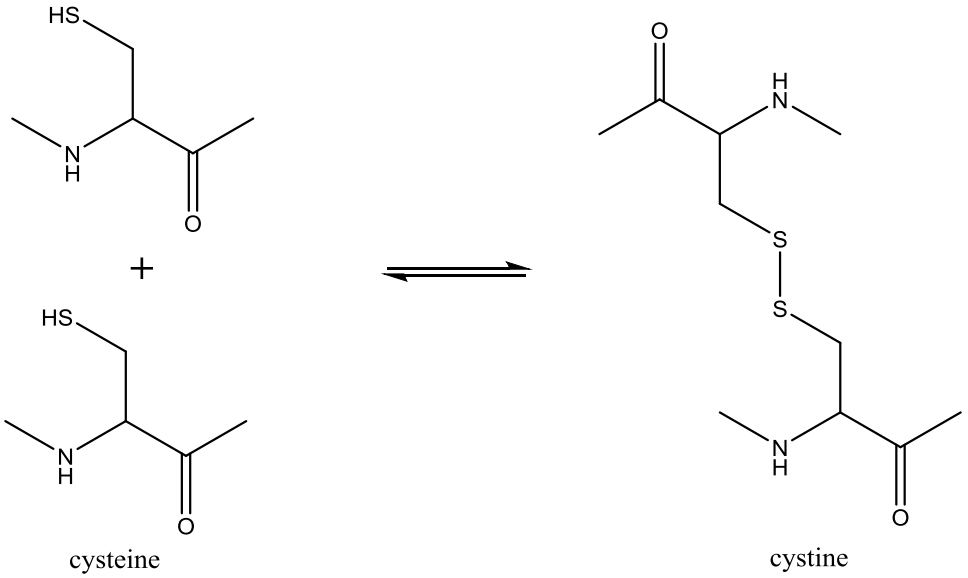
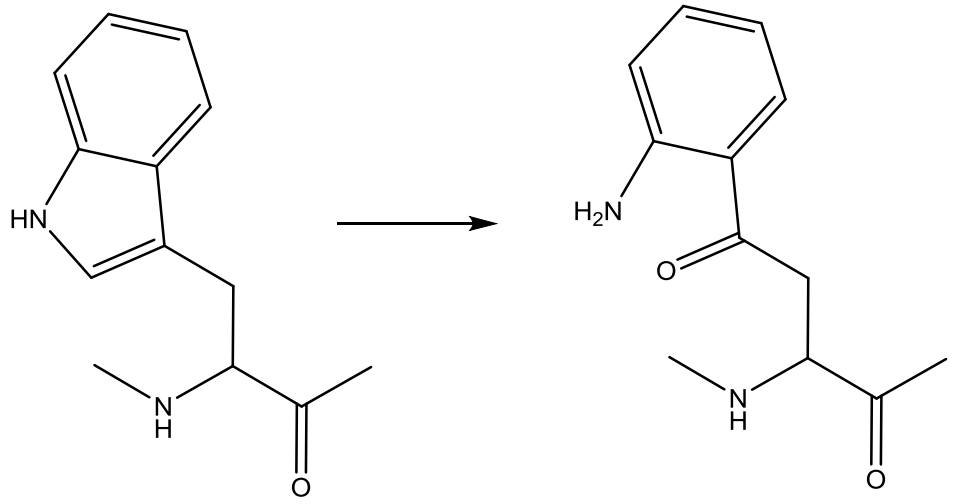


Figure 9 The oxidation of cysteine to cystine



tryptophan kynurenine

Figure 10 The oxidation of tryptophan to kynurenine.

In addition to the theoretical properties as discussed above, the peptides practical assets must be considered; they should give chromatographic separation and high MS sensitivity. While laboratory analysis could give answers to these questions, several databases list in-silico cleaved peptides known to meet the practical demands. Examples of such databases are PeptideAtlas [53-55] and the proteomics identifications database (PRIDE) [56, 57]. The enhanced signature peptide (ESP)-predictor [58] was developed to predict peptides providing high MS-response [59], while the open-source software for targeted proteomics, SKYLINE [60], is a database for establishment of methods (choosing peptides and transition optimization) and analysis of data [61].

3.2.3 Electrospray ionization

The MS demands the analyte ion to be in gaseous phase, something that could be a challenge as proteins and peptides are large, non-volatile species. To solve this problem electrospray ionization (ESI) is used (**Figure 11**). ESI converts analyte ions from liquid into gas phase, and is easily coupled to a liquid chromatography system, making it a preferred choice when analysing complex protein/peptide samples. The ions remain intact, i.e. are not fragmented, hence it is a soft ionization method.

In ESI, the mobile phase (MP) with analyte is sprayed out of a capillary outlet, where a potential is applied. Small droplets with high charge are produced, giving an electrically

charged spray. From the Taylor cone, which the capillary liquid tip is called, droplets fissions into smaller and smaller droplets. Two main models are used to describe what is happening at the end of the spray, where the droplet ions are converted to gas phase ions; the ion evaporation model (IEM) [62] and the charge residue model (CRM) [63]. The IEM explanation is that when the droplet reaches a certain size, the repulsive forces between the ions in the droplet becomes greater than the surface tension of the droplet. When this is the case, a droplet containing one analyte ion leaves the "parent" droplet, and gas phase ions are formed after final evaporation. This model favour small molecules. CRM explains the gas formation with fission; droplet evaporates to a certain size and then fissions into smaller droplets. This continues until single – ion droplets are formed, that can evaporate giving gas – phase ions.

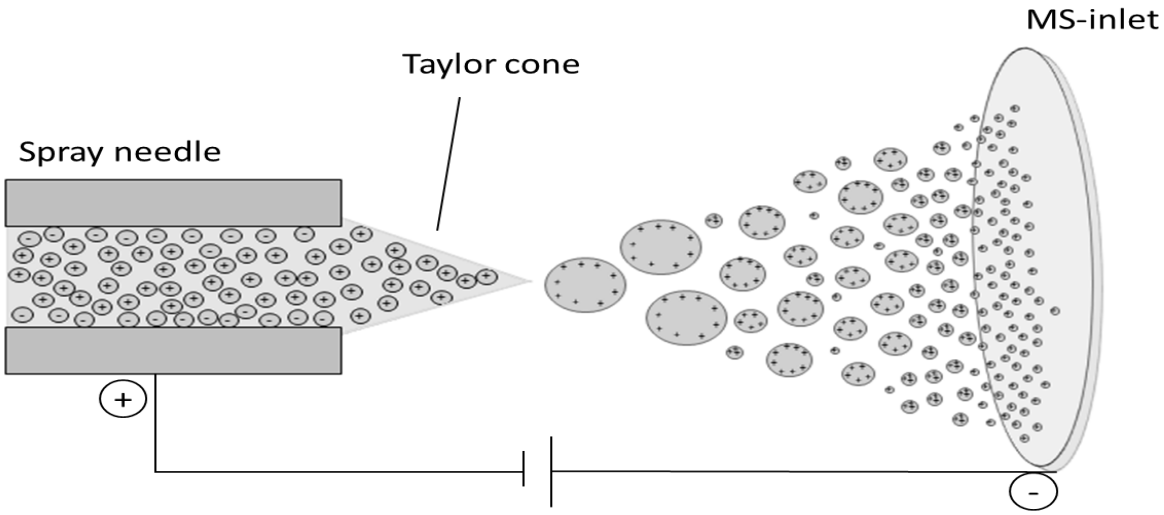


Figure 11 Electrospray ionisation (ESI) in positive mode. *A voltage is applied to the needle (positive in positive mode), attracting the negative ions to the walls, and to the MS-inlet (negative in positive mode), attracting the positive ions. A spray is formed at the end of the Taylor cone, and droplets fissions into smaller and smaller droplets. Two different theories describe the gas-formation taking place at the end of the spray; the ion evaporation model and the charge residue model.*

A challenge with the ESI interface is possible ion suppression. Ion suppression happens when several ions compete to be at the droplet surface. If an ion is high-abundant, surface-active and enters the ESI with the analyte, this ion will hinder the analyte ion to be at the droplet surface, and hence suppress the formation of analyte gas-phase ions. ESI-MS is a

concentration sensitive detector and flow dependent, meaning that if the liquid sample enters the spray with a low flow, a higher amount of the ions will be transferred into the gaseous phase, increasing sensitivity [64-66].

3.2.4 Liquid chromatography

Even though MS provides high selectivity, analytes must be separated in order to avoid ion suppression (giving better sensitivity) and to increase selectivity when analysing complex samples.

In column chromatography, compounds are separated on a column, which is filled with a stationary phase (SP). Based on their affinity toward the SP, the compounds (analytes) will be retained. Analytes with high SP affinity will use more time before it reaches the detector. Based on the properties of the analytes, liquid or gas chromatography is used. With gas chromatography (GC), the MP which transport the analytes through the column, is a gas. When using GC, analytes must be volatile and thermally stable, as they are brought to gas phase before entering the column. Analytes not suitable for GC, such as peptides, are separated using liquid chromatography (LC), which is used in this thesis. The MP is a liquid and analytes are kept in the liquid phase when separated on the column, before transferred to the gaseous phase in the electrospray.

In reversed-phase (RP) LC, the SP is hydrophobic (and MP is aqueous) and the analytes are retained on the column based on their hydrophobicity. Analytes with high hydrophobicity will spend more time on the column and have a longer retention time than more hydrophilic analytes. The most used stationary phase is silica based C18 particles. In conventional LC (inner diameter (i.d.) 2-5 mm) the particle size ranges between 1.7 and 5 µm, and column lengths are between 5 to 30 cm.

Nano LC

When analyte abundance is low, a high sensitivity is needed. As ESI sensitivity/ion suppression is flow dependent, a chromatographic system with lower flow could provide improved detection; nano LC. Nano LC is a miniaturized chromatographic system with a column i.d. of 50-100 µm and a MP flow rate of 20-500 nL/min, compared to 2-5 mm and 0.5-2.5 mL/min for conventional systems. Particle size and length are the same as with conventional columns; 2.4-5 µm and 5-30 cm, respectively. Nano columns give a lower radial

dilution [66] (***Figure 12***), enhancing the sensitivity for concentration sensitive detectors, such as the ESI-MS. The back pressure and time of analysis is the same as for conventional system due to same linear flow rate, but the MP consumption is less.

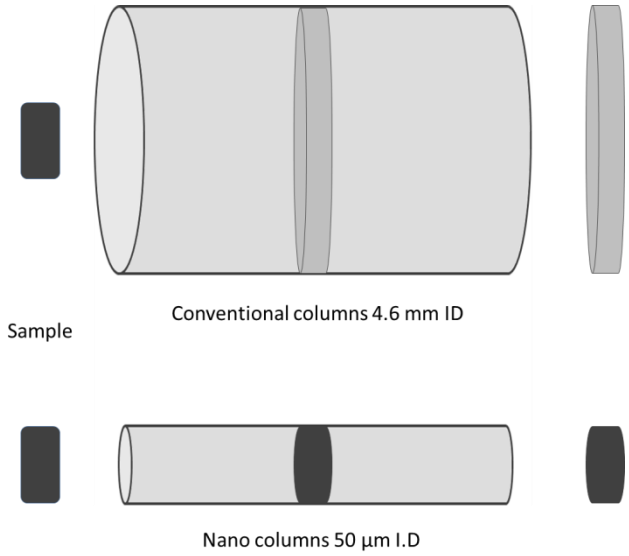


Figure 12 Sample dilution using conventional LC and nano LC. *Samples applied to a nano LC system, where the column inner diameter is reduced by approximately a 20 fold compared to a commercial LC-system, are less diluted by the mobile phase, providing enhanced sensitivity for concentration sensitive detectors. Colour intensity indicates concentrations of an analyte. Figure is adapted from [66].*

WB has in the past been the superior compared to LC-MS regarding protein quantification, but questions are now raised, concluding that "it is time to turn the tables" [67]. Where WB is very much dependent on the specificity of one antibody and the intensity of the band, LC-MS depends on several parameters; retention time and *m/z*-ratio of both precursor and fragment ions. In addition several peptides could be detected per protein. The WB method is still useful (and used in this thesis), also in LC-MS method development when the detection of the protein is not established. WB is also a good tool for verifying the success of e.g. a protein enrichment step.

Established techniques

The role of CYP27a1 as a novel participant in human cancer is established, but methods for the determination of CYP27a1 levels, needed to understand e.g. the development of ER+ breast cancer are not fully assessed, as should be. Most published work relies on the determination of CYP27a1 products (e.g. 27-OHC [68]), the use of immunobased methods (e.g. WB [68, 69]), or other methods (e.g. luciferase activity [70] and RNA measurements

[71]). Determination of CYP27a1 by LC-MS has been established for analysis of human retina [72], where the protein is quantified based on two signature peptides, but not for cells.

3.3 Preparation of biological samples

Even though todays chemical methods ensure sensitive and selective analysis of complex biological samples, sample clean-up is still necessary. Biological experiments, e.g. using antibodies, are important tools during development of a sample preparation method for MS analysis. In this work several cell and bioanalytical experiments were used and all of them are described in this section.

3.3.1 Cell culturing

When targeting proteins (or other components of a cell), growing cells in vitro – cell culturing – is a way to produce the analyte outside its natural environment, providing a non-invasive availability of the analyte. By growing cells outside their natural environment, a cells behavior and response to controlled influences could be studied. Different cell lines are used based on the goal of the study; e.g. when studying breast cancer, the breast cancer cell lines Michigan Cancer Foundation 7 (MCF7) and MDA-MDB-231 are often used. A breast cancer cell line is derived from the breast cancer tissue and the cells remains with the same traits as they possessed in their natural environment (the MCF7 cell line was isolated from a woman in 1970 [73]). A more detailed description of the practical cell culturing work is described in ***section 9.1.1*** in appendix.

3.3.2 Transfection

When the protein abundance is low, or the antibodies show poor selectivity, transfection could be applied in the workflow. Transfection, first described in the mid-60s [74, 75], is a method for transporting nucleic acids, RNA or DNA as in this study, into a cell [76]. Modified cells are produced during cell division; hence the cells are forced to produce the protein coded for by the DNA. The implemented DNA used in this work codes for the target protein, CYP27a1, tagged with an extra amino acid sequence, DYKDDDDK[1] ***(Figure 13)*** (called FLAG-tag) [77]. This tag enables the use of immuno-based methods when the performance of the antibodies of the targeted peptides are limited [78].

[1] Amino acids are aspartic acid (D), tyrosine (Y) and lysine (K).

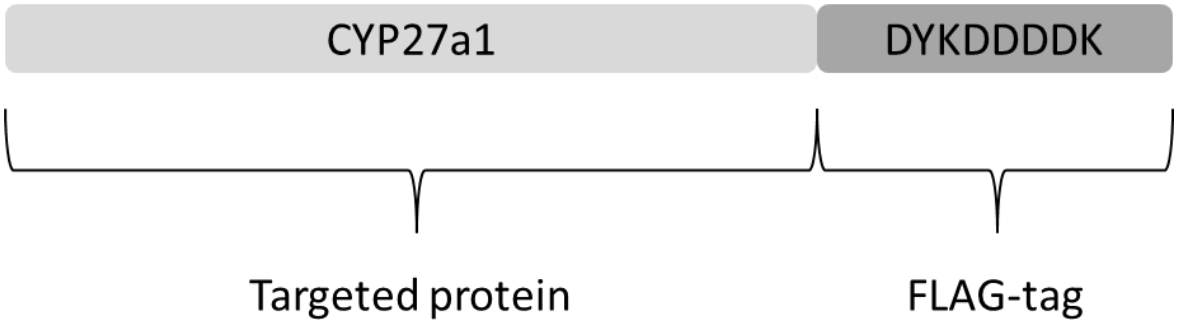


Figure 13 The CYP27a1–FLAG protein. *By using transfection, DNA encoding for a protein is implemented into a cell. When cells divide, regulated cells are produced, containing the new protein or elevated levels of an existing protein, based on the implemented DNA. In this thesis the DNA coding for the CYP27a1-protein with an additional amino acid sequence (DYKDDDDK), called FLAG, was used.*

If the DNA is integrated in the cell nucleus, stable transfection is achieved, and will survive even during cell division. However, if the DNA is only implemented in the cell, but not the cell nucleus, a transiently transfection is achieved; during proliferation, the DNA amount will be diluted, making the transfection effect to only be present for a certain amount of time. How to produce the DNA and insert it into the cells is described in ***section 9.1.2*** in appendix.

3.3.3 Immunoprecipitation

Protein samples are often complex, and a clean-up step is beneficial to reduce sample complexity. Immunoprecipitation (IP) [79] uses antibodies (***Textbox 3)*** coupled to magnetic beads (agarose, a polymer, could also be used when sample volume is larger than 2 mL) to "fish" the target protein from the sample. ***Figure 14*** shows an overview of the IP procedure. The magnetic beads are covered with protein A [80] or G [81, 82] based on the origin of the antibody. The proteins binds specific to an antibody based on the origin and type of antibody (e.g. IgG or IgM). For example, protein G has higher affinity for an IgG antibody produced in rabbit, compared to an IgG antibody produced in rat [81, 82]. When an antibody is added to the magnetic beads, the heavy chain-only part of the antibody binds to the protein A or G, and hence to the magnet. This leaves the antigen binding site of the antibody exposed for protein binding. Also, other targets than a single protein could be addressed; e.g. Co-IP which is used for protein complexes (when binding to the protein, the entire complex is precipitated), and chromatin IP (Chir-IP) which targets DNA-binding proteins to be able to precipitate the DNA which the protein is in a complex with.

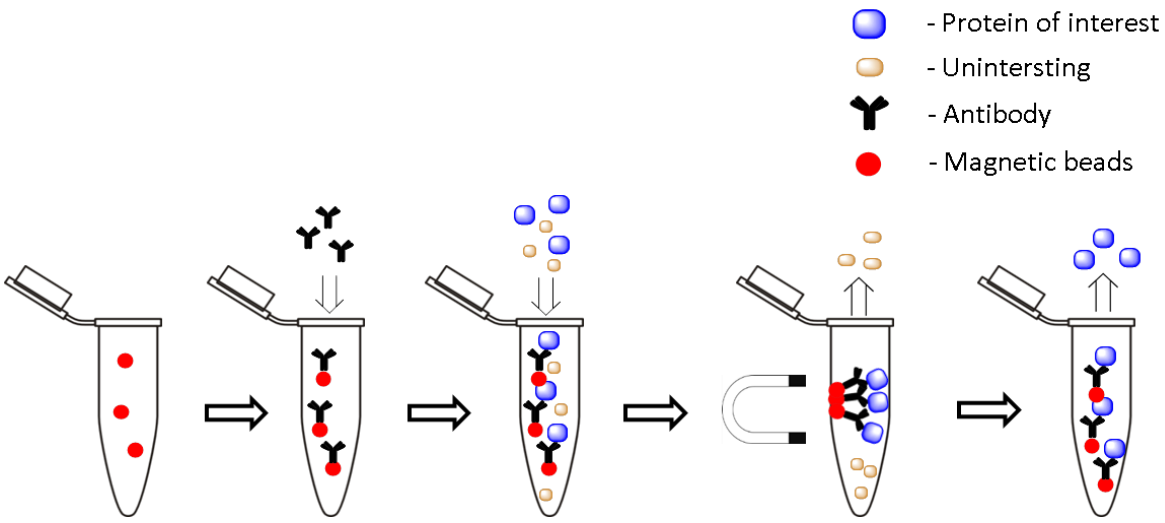


Figure 14 The procedure for immunoprecipitation. *The antibodies are first coupled to the magnetic beads, before the protein sample is added. The antibodies subsequently connect to the target protein, giving a bead-antibody-protein-complex. By using an external magnet the rest of the protein sample can be removed, leaving only the complex containing the target protein in the tube. The target protein is then subsequently eluted from the antibody-bead-complex.*

3.3.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Another sample-clean up step, which is not dependent on antibodies, is gel electrophoresis (GE, the first step in WB). This method separates proteins (also DNA and RNA, but will not be described in this thesis) based on their size. In order to do so, the protein samples are applied on a porous gel. The sodium dodecyl sulfate polyacrylamide gel electrophoresis method (SDS-PAGE [83-85]), is widely used for separating proteins, and is applied in this thesis. In SDS-PAGE, the gel is made up by polyacrylamide (when applying other GE methods, e.g. agarose could be used as gel), and SDS is added to the samples prior to application on the gel. SDS is a detergent (***Textbox*** 2) that makes adducts with the proteins giving all proteins the same charge /size ratio and that will move with the same speed at the applied voltage. However due to the pores in the gel the proteins will be separated according to their size. The speed of

Textbox 2 Detergents

Detergents

A compound that functions like soap; it is amphiphilic (has one polar and one unipolar side), and in reaction with proteins the non-polar side binds to the protein, leaving the polar, charged end of the detergent exposed. This gives rise to a total charge, which is taken advantage of in e.g. gel electrophoresis. Examples of detergents are SDS and urea.

the proteins is inversely proportional to the size, making smaller proteins travel further through the gel holes than the larger ones, at a defined period of time.

4 Aim of study

Originally this study aimed at studying the role of Gli 1, Gli 2 and Gli 3 and the oxysterol related CYP enzymes CYP27a1, CYP46a1 and OH25 in the Hedgehog signaling pathway. However, preliminary investigations showed that it was difficult to identify these proteins by the available methods; hence the focus was shifted towards only CYP27a1 and its relation to breast cancer. Thus, the aim of the thesis is to investigate sample preparation procedures of cell samples and subsequently establish a nano LC-MS method for detection of CYP27a1.

5 Materials and methods

Laboratory work was performed at both Oslo University Hospital (OUS) and the Department of Chemistry (KI), where equipment and chemicals are different or have different suppliers. Sections where this is of importance are marked with either KI or OUS. Where it is needed in order to find the correct product, the catalogue number is mentioned.

5.1 Materials

5.1.1 Chemicals (KI)

Acetonitrile (ACN) HiPerSolv Chromanorm® for LC-MS was from VWR (Radnor, PA, USA). Formic acid (FA, for MS,~ 98 %), trifluoroacetic acid (TFA, for HPLC (high pressure liquid chromatography), ≥ 99.0 %), ammonium bicarbonate (ABC, ReagentPlus®, ≥ 99.9 %), DL-dithiothreitol (DTT, Bioultra, ≥ 99.0 %), iodoacetoamide (IAM, Bioultra, ≥ 99.0 %) and urea (98 %) were all from Sigma Aldrich (Sigma, St. Louis, Missouri, USA). Optima® LC-MS water used for mobile phases was from Thermo Scientific (Thermo, Waltham, Massachusetts, USA). Type 1 water was produced using the Milli-Q® integral water purification system connected to a Q-POD® element with a Millipak® express 40 filter (0.22 µm), all from Millipore (Billerica, Massachusetts, USA). Trypsin/Lys-C-mixture (V5071) was from Promega Biotech AB (Madison, Wisconsin, USA).

5.1.2 Chemicals (OUS)

RPMI (Roswell Park Memorial Institute) 1640 growth medium (R8758) was from Sigma. Gibco® Dulbeco's modified eagle medium F12 (DMEM, 21041-025) was from Thermo. Eagle's minimum essential medium (EMEM) was from ATCC® (American Type Culture Collection, 30-2003™, Manassas, Virginia, USA). Gibco® fetal bovine serum (FBS, 10270) and Gibco® insulin-transferring-selenium (ITS, 100x) were both from Thermo, and penicillin/streptomycin (P/S) was from Sigma. The phosphate-buffered saline (PBS) was prepared by the Department of Microbiology at Ullevål University Hospital (MIK, Oslo, Norway). Tween® 20 (P1379, viscous liquid), trypsin-ethylenediaminetetraacetic acid (EDTA)-solution (T3924) and dimethyl sulfoxide (DMSO, BioReagent, for microbiology, ≥ 99.9 %) were from Sigma. Information about cells lines used and associated information is shown in ***Table 1*** below. All cell lines were from ATCC®.

Table 1 Overview of cell lines. *Six different cell lines were used in this work, with their different origin and growth medium.*

Cell line	ATCC® product number	Origin	Growth medium
BxPC-3	CRL-1687™	Human pancreatic adenocarcinoma	RPMI 1640
HEK293	CRL-1537™	Human embryonic kidney (no disease)	RPMI 1640
RKO	CRL-2577™	Human colon carcinoma	EMEM
MCF7	HTB-22™	Human mammary gland, breast, adenocarcinoma Estrogen receptor positive	DMEM
MDA-MB-231	HTB-26™	Human mammary gland, breast, adenocarcinoma Estrogen receptor negative	DMEM

1 M tris (pH 8) was from MIK. Tris-HCl (RES098T), sodium chloride, EDTA (99.995 % trace metals basis), glycerol (for molecular biology, ≥ 99 %), trizma® base (≥ 99 %, T1503), glycine (ReagentPlus®, ≥ 99 %, HPLC), SDS (≥ 98.5 %, L3771), citric acid monohydrate (C1919), urea (electrophoresis reagent) and bromophenol blue sodium salt (B5525) were all from Sigma. Gibco® 2-mercaptoethanol (50 mM) was from Thermo and water was purified by a Direct-Q® (DQ) water purification system from Millipore. Technical methanol (20903.368) was from VWR. The protease inhibitor cocktail tablets (cOmplete mini, EASYpack) were from Roche (Basel, Switzerland). The magnetic bead solution (Dynabeads® protein G, 10003D) used in IP and NP-40 (nonyl phenoxypolyethoxylethanol) used in cell lysis buffer (FNN0021) were from Thermo. The Coomassie brilliant blue R-250 staining solution was from Bio Rad. The Emsure® acetic acid (glacial) 100 % was from Merck. Antibodies used in IP and WB with relevant information are listed in Table 2 below.

Table 2 Overview of antibodies. *List of antibodies used in this work, with origin, supplier, product number, concentration and application. Concentrations are listed only for antibodies used for IP.*

Antibody	Origin	Number	Supplier	concentration	Used in
CYP27a1	Goat polyclonal	SC-14835	Santa Cruz Biotechnology Inc, Santa Cruz, California, USA)	200 µg/mL	IP
CYP27a1	Mouse polyclonal	ab-89781	Abcam, Cambridge, United Kingdom	1000 µg/mL	IP
CYP27a1	Rabbit polyclonal	APO6773PU-N	Acris Antibodies.	1000 µg/mL	IP, WB
Beta-catenin	Mouse polyclonal	610153	BD transduction laboratories^{TM}. BD Biosciences, Franklin Lakes, New Jersey, USA	250 µg/mL	IP, WB
Anti-flag	Rabbit polyclonal	F7425	Sigma	800 µg/mL	IP, WB
Anti-rabbit	Donkey polyclonal	Sc-2313	Santa Cruz		WB
Anti-mouse	Donkey polyclonal	Sc-2314	Santa Cruz		WB
Actin	Rabbit polyclonal	A2066	Sigma		WB
Normal goat IgG	Goat	Sc-2028	Santa Cruz	400 µg/mL	IP
Normal Rabbit IgG	Rabbit	Sc-2027	Santa Cruz	400 µg/mL	IP
Normal mouse IgG	Mouse	SC-2025	Santa Cruz	400 µg/mL	IP

The CYP27a1-FLAG plasmid (VB150311-10002) was from Cyagen Biosciences (Santa Clara, California, USA), and the pEGFP-C1 plasmid (6084-1, enhanced green fluorescent protein, EGFP) was from Clontech Laboratories Incorporated (Mountain View, CA, USA, member of Takara Bio Incorporated, Kusatsu, Shiga, Japan). Both the lysogny broth (LB) culture dish with ampicillin (AMP) and the LB-medium with AMP were from MIK-OUS. JETSTAR 2.0 midi-prep kit from Genomed Gmbh (Löhne, Germany) was used for DNA extraction. The Fugene® HD transfection reagent was from Promega. Opti-MEM® reduced serum medium, the 3-8 % tris-acetate gels (NuPAGE®), the PageRuler™ prestained protein ladder and the tris-acetate SDS running buffer (NuPAGE®, 20x, LA0041) were from Thermo. Non-fat dried milk (A0830,0500) was from AppliChem, and the tris buffered saline tween (TBS-T) tablets were from Medicago AB (Uppsala, Sweden). WB-bands were visualized using ECL™ (enhanced chemiluminescence) prime western blotting detection reagent (RPN2236, Ge Healthcare)

5.1.3 Equipment/instrumentation (KI)

The Speed-Vac™ SC110 concentrator was from Savant (now Thermo). 1.5 mL vials, protein LoBind tubes 1.5 mL and centrifuge (5415 R) were all from Eppendorf (Hamburg, Germany), while the thermoshaker (PSC-20) was from Grand Instruments (Cambridge, United Kingdom). To desalt peptides, a 100 mg and 3 mL C18 isolute solid phase extraction (SPE) column from Biotage (Uppsala, Sweden) was used. Protein concentrations were measured using NanoDrop 2000 UV-Vis spectrophotometer (Thermo) at 205 nm. All LC-experiments were performed on an EASY nanoLC 1000 pump from Thermo. 0.3 mL plastic snap ring vials (548-0120) with belonging snap ring caps (548-0893), used in the auto injector, were from VWR. The fused silica capillary with 20 µm i.d. was from Polymicro Technologies (Phoenix, Arizona, USA). Precolumns used were; an Acclaim PepMap™ 100 precolumn (75 µm x 2 cm, NanoViper, C18, 3 µm particle size, and 100 Å pore size) from Thermo, a silica-based C8 monolithic precolumn (5 cm x 50 µm) produced in-house by Tone Smetop [86] and a polystyrene divinylbenzene (PS-DVB) precolumn (5 cm x 50 µm) produced in-house by Tore Vehus [87]. The C18 analytical column, 15 cm fused silica (Polymicro technologies) with 50 µm i.d. and 2.6 µm Accucore particles (80 Å pore size) from Thermo, was packed in-house by Henriette Sjånes Berg [88]. The LC-column was coupled to a Q Exactive™ Hybrid Quadrupole Orbitrap mass spectrometer, equipped with a nanoFlex nanospray ion source, using a stainless steel nanobore emitter (ES542) with 20 µm i.d., both from Thermo. An

Upchurch PEAK microtight® fitting from Sigma was used to connect the column and the emitter. Both data processing programs, Proteome Discoverer (v1.4) and XCalibur (v2.1) were from Thermo.

5.1.4 Equipment/instrumentation OUS

All cell work was performed in a Scanlaf Mars Safety Class 2 (Labogene, Lynge, Denmark) hood. Cells were grown at 37° in a humidified atmosphere containing 5 % CO_2 (Forma Steri-cycle CO_2 Incubator), using Nunc™ cell culture treated EasYFlasks™, both from Thermo. The Falcon™ 6 well tissue culture plate used for transfection was from Corning Incorporated (Corning, NY, USA). For cell storing, CryoTube™ vials and Mr. Frosty™ freezing container (both Thermo) were used. Both the Axiovert 25 microscope used for cell culturing work and the Axiovert 200M equipped with AxioCam (pictures taken using AxioVision) used for transfection evaluation were from Zeiss (Oberkochen, Germany). The water bath was from Julabo (Seelbach, Germany), and the TC™ automated cell counter was from Bio Rad (Hercules, California, USA). The centrifuge (5810 R) used was from Eppendorf, but for centrifugation at 12000 g, a microcentrifuge (type 157) from Ole Dich Instrumentmakers ApS (Hvidovre, Denmark) was used. The magnet used for IP was from Invitrogen, Thermo. 1.5 mL vials (Labdesk, 20170-038) and 15 and 50 mL tubes were from VWR. The rotators used for IP incubation were from Labinco B.V (when room temperature, 20170-038, Breda, the Netherlands) and Bibby Scientific (when 4 °C, S83, Stuart, Stone, Staffordshire, United Kingdom). The roller used for WB was also from Stuart. Incubation at 37 °C was performed in an incubator from Termaks (Bergen, Norway). DNA concentration was measured using Ultrascpec 2100 pro from Amersham Biosciences (now GE Healthcare, Buckinghamshire, UK). Protein concentration was measured using BCA (bicinchoninic acid) protein assay kit (Pierce™, Thermo), on a FLUOstar Omega microplate reader (Isogen Life Science, De Meern, the Netherlands). The electrophoresis chamber (Novex® Midi Cell) was from Thermo. Pipette tips with a microcapillary tip (732-0508) were from VWR. Proteins were transferred to a 0.2 µm polyvinylidene fluoride (PVDF) membrane (Immobilon®-P^{SQ}, ISEQ00010, Merck) using filter paper (extra thick block paper, 1703960) and a trans-blot SD semi-dry transfer cell from Bio-Rad. Bands were visualized on an overhead projector (OHP) transparency film (Nobo, integrated part of ACCO Brands Corporation, Lake Zurich, Illinois, United States) in a Chemidoc™ touch imaging system (Bio-Rad). WB raw-files were processed in Image lab software (Bio Rad).

5.2 Solutions

If nothing else is mentioned, the water used is type 1 water (KI) or DQ water (OUS).

5.2.1 KI

25 mM ABC was made by dissolving 0.10 g ABC powder in 50 mL water. 50 mM ABC was made by dissolving 0.20 g ABC in 50 mL water. These solutions were freshly made before each use. 8 M urea was made by dissolving 48.05 g urea (mm = 60.06 g/mole) powder in 100 mL type 1 water. 500 mM IAM and DTT were made by dissolving 0.23 g IAM (mm = 184.96 g/mole) powder and 0.19 g (mm = 154.25 g/mole) DTT powder, respectively, in 2.5 mL 8 M urea each. Before use, 4 µL of 500 mM DTT or IAM were added to 196 µL 25 mM ABC, giving 10 mM DTT/IAM-solutions. A 20 ng/µL trypsin/Lys-C solution was made by adding 1 mL of ABC to a volumetric glass flask containing 20 µg of the trypsin/Lys-C mix. Aliquots of 100 µL of the IAM, DTT and trypsin/Lys-C-solutions were frozen at -80 °C. These solutions were thawed right before use, and any leftovers were thrown. To make 0.1 % TFA solutions, 100 µL TFA were dissolved in the respective liquid, type 1 water or ACN, to a total of 100 mL. 2 % ACN in 0.1 % TFA was made by mixing 2 mL ACN and 100 µL TFA with type 1 water to a total of 100 mL. MP A consisted of 0.1 % FA in LC-MS water, and MP B consisted of 0.1 % FA in ACN. Both were made by adding 100 µL of FA in 50 µL of water or ACN and adjusted to a total volume of 100 mL. The 5 % FA + ACN (1 + 2) solution was made by adding 100 µL of FA and 9.9 mL of water to a 50 mL tube, and subsequently filling it to the 30 mL mark with ACN.

5.2.2 OUS

All cell growth media (500 mL) were supplemented with 50 mL PBS (10 %) and 10 mL P/S (2 %). RPMI 1640 medium was also supplemented with 500 µL ITS. PBS with 0.1 % Tween® 20 (PBS-T) used for IP was made by adding 50 µL of Tween® 20 to a 50 mL vial followed by 50 mL PBS and vortexing. This solution was stored at room temperature. TBS-T used in WB was made by adding 10 TBS-T tablets into a 5 L glass flask and filling it to the 5 L mark with water. 5 % and 1 % milk-solutions were made by adding 25g and 5 g, respectively, of dried milk powder to a 500 mL glass flask, and filling to the 500 mL mark with TBS-T-solution. These solutions were stored at 4 °C for a few weeks (until the milk started to smell). When making diluted antibody solutions, antibody was added to the vial

before adding the designated milk solution, see ***Table 3***. For example, when making a 6 mL 1:500 of antibody in 1 % milk in TBS-T-solution, 12 µL of the antibody solution was added to a 50 mL vial followed by 6 mL 1 % milk in TBS-T. The antibody solutions were kept at -20 °C and were reusable.

Table 3 Antibody dilution factors and solutions used in WB.

Antibody	Dilution factor	Diluted in
CYP27a1	500	1 % milk in TBS-T.
anti-FLAG	500	1 % milk in TBS-T.
anti-actin	2000	5 % milk in TBS-T.
Beta-catenin	10 000	5 % milk in TBS-T.
anti-rabbit	10 000	5 % milk in TBS-T.
anti-mouse	10 000	5 % milk in TBS-T.

NP-40 lysis buffer was made by mixing the components (***Table (AP) 1***) in a 50 mL vial. It was frozen at -20 °C in 10 mL aliquots. When needed, one 10 mL aliquot was thawed, and a protease inhibitor tablet was added before vortexing, and aliquoting into 1 mL vials, which were stored at – 20 °C (stable for a few months). As long as the NP-40 lysis buffer with protease inhibitor solution was kept on ice when thawed, it was reusable.

To make the 5x loading buffer to be mixed with samples before doing electrophoretic separation, the components (***Table (AP) 2***) were mixed using set amounts. It was frozen at – 20 °C in 500 µL aliquots. Before use it was thawed and diluted to 1x by adding it to the samples.

The 10x transfer buffer used in WB to transfer proteins from the gel to the membrane was made by mixing components and their respective amounts (***Table (AP) 3***). When making 1x buffer, 100 mL of the 10x buffer were mixed with 200 mL methanol and 700 mL water. Both buffers were stored at room temperature.

Fixation buffer for gels prior to gel digestion contained 50 % methanol, 10 % acetic acid and 40 % water. The buffer was made by adding 500 µL of methanol to 300 mL of water. 100 mL of acetic acid was added to the solution and water was used to adjust the total volume to 1L.

5.3 Biological methods

5.3.1 Cell culturing

When starting a new cell line, frozen cells were thawed, followed by centrifugation (800g, 5min) to pellet cells so solvent could be removed. Cell pellet were suspended in the cell growth medium, transferred to the culturing flask and diluted with growth medium. See ***Table 4*** for more details about flask type used based on number of cells, and respective liquid amounts. When confluent, the cells were first washed with PBS, by carefully swirling the flask. PBS solution was then removed, and trypsin/EDTA solution was added to the flask, covering the cells, to loosen them from the flask. Cells were then incubated at 37 °C in a humidified atmosphere containing 5 % CO_2 until cells were loose from flask surface, and subsequently mixed by re-suspension. Cells in trypsin solution were then split, growing further the small part, and freezing the large part. The small part was added to the cell culturing flask and growth medium was added to the total volume suited for flask size. Before freezing the large part, medium was added to cell solution (how much is not important, normally 3-10 mL) to terminate the trypsination. The number of cells was measured by pipetting 10 µL of the solution onto a cell counter glass, which was inserted into the cell counter, which reported the number of cells per mL cell solution. Subsequently, the remaining cell solution was centrifuged to make cell pellet. All liquid was removed and the cell pellet was re-suspended in PBS, before further centrifugation. This washing step was repeated once more before cell pellet was resolved in suitable amount of NP-40 lysis buffer and frozen at -80 °C for further analysis. When freezing down cells suited for further growth later, the PBS-washed cell pellet was diluted in 900 µL medium with 100 µL DMSO in a CryoTube™ vial. This was stored in a Mr. Frosty™ freezing container at -80 °C for a day to ensure slow freezing, before the tube was transferred to a nitrogen tank (-200 °C).

Table 4 Amount of components for cell culturing based on number of cells. *Three different cell culturing flask sizes were used based on number of cells that were in culture. Amount of PBS, Trypsin/EDTA solution and growth medium was based on flask size.* [a]*Number of cells when confluent will vary according to cell types. The listed values are approximate values.*

Number of cells when seeding	Cell flask culture area	Number of cells at confluency[a]	PBS	Trypsin/EDTA	Medium
$0.7 * 10^6$	25 cm^2	$2.8 * 10^6$	3 mL	3 mL	3-5 mL
$2.1 * 10^6$	75 cm^2	$8.4 * 10^6$	5 mL	5 mL	8-15 mL
$4.6 * 10^6$	175 cm^2	$18.4 * 10^6$	10 mL	10 mL	15-30 mL

In order to grow more cells, the entire trypsin/EDTA cell solution (after trypsination and subsequent re-suspending) was pipetted into a larger flask, and medium was added to appropriate volume.

5.3.2 Immunoprecipitiation

To connect antibody to beads, the bead solution was vortexed and 50 µL were transferred to a vial. Supernatant was removed by placing the vial on the magnet, letting the beads be attracted to the magnet. 3 µg of antibody solution (µL calculated according to antibody concentration, see ***Table 2***) were solved in 200 µL PBS-T and transferred to the vial containing the beads. The bead-antibody solution was re-suspended, before 1 hour incubation at room temperature with rotation. Supernatant was removed and 1 mg of protein (cell lysate) was added to the bead-antibody solution, and allowed to react overnight at 4 °C with rotation to form a bead-antibody-protein complex. The following day, the supernatant was removed, and the bead-antibody-protein complex was washed by re-suspending carefully three times with 200 µL PBS (the supernatant was discarded between washes). The bead-antibody-protein complex was resolved in 100 µL PBS, transferred to a new vial, and the supernatant was discarded. To elute proteins from the beads, 25 µL of 0.2 M citric acid were added to the vial and incubated for 5 minutes. The supernatant was transferred to a new vial. This step was repeated twice, and supernatants were combined. The extract was now ready for further use.

5.3.3 Transformation

200 µL of the bacterial solution were pipetted onto a LB culture dish with AMP and incubated overnight at 37 ºC in order to form bacterial cultures. One or two cultures were scraped off, mixed with 50 mL LB medium with AMP, and the solution was incubated overnight at 37 ºC. After incubation, the solution was transferred to a 50 mL vial and cells were pelleted by centrifugation. The medium was completely removed. DNA was extracted from bacterial cells using the protocol of the JETSTAR 2.0 midi-prep kit. All solutions used, referred to as solution 1-6, came with the kit. In each step the column was allowed to empty by gravity flow. Columns were first activated by applying 10 mL of solution 4. As the solution run through the column, 4 mL of solution 1 were added to the cell pellet, followed by re-suspending. Subsequently solution 2 was added to the solution for cell lysis and mixed gently by inverting. The solution was kept on the bench for 5 minutes before 4 mL of solution 3 were added for neutralization. It was mixed immediately by multiple inverting. When the solution became homogenous it was centrifuged at 800g at room temperature for 10 minutes. The supernatant was applied to the column (no solution 1 left in the column). The column was washed by adding 2 x 10 mL of solution 5. The DNA was eluted from column by applying solution 6. When all solutions had completely eluted, 3.5 mL of cold isopropanol was added to the solution to precipitate the DNA. The solution was subsequently centrifuged at 12 000g for 30 minutes, and the supernatant was carefully removed by quickly inverting the tubes. 3 mL 70 % ethanol were added to the pellet, and the solution was centrifuged for 10 minutes at 12 000g. Again the supernatant was carefully removed by quickly inverting the tube. The tube with the cell pellet was allowed to dry by keeping the tube up-side down. The cell pellet was dissolved in 10 µL 10 mM Tris-HCl (pH 8) and transferred to a 1.5 mL vial. The DNA concentration was measured by diluting 2 µL of the DNA solution in 98 µL water. The spectrophotometer was set to DNA measurements, with 10 mm path length, 380 nm corrections, and a dilution factor of 50. Water was used as reference, and concentrations were reported in µg/µL. The solution was stored at -20 °C.

5.3.4 Transfection

600 000 cells per well were cultured in a 6-well plate, following same procedure as in ***section 5.3.1***. When cells had reached 40-70 % confluence, the medium was replaced by growth medium without P/S and ITS and incubated for one hour. For each well, 2 µg of DNA (based on DNA concentration found in ***section 5.3.3***) were mixed with 200 µL optimum medium in a

1.5 mL vial and vortexed, before further mixing with 6 µL Fugene HD reagent (µg DNA + µL Fugene HD, 1 + 3). The solution was vortexed and incubated for 15 minutes at room temperature, before it was evenly dripped by a pipette onto the cells. Cells were incubated at 37 ºC overnight. On the following day, the medium was changed to cell growth medium with FBS, P/S and ITS. For positive control, the EGFP-DNA was used in one well. In another well, all reagents were added except DNA, in order to function as negative control. Transfection was evaluated using a fluorescent microscope as the EGFP protein (produced in the well where EGFP-DNA was added) emits fluorescent light. When cells were confluent, they were frozen following the procedure with PBS washing and trypsination already described in ***section 5.3.1***.

5.3.5 Western blotting

The cell lysate was diluted to a protein concentration of 1 µg/µL. For each sample to be analysed with WB, 24 µL of cell lysate were mixed with 6 µL 5x loading buffer, re-suspended, and heated at 70 °C for 10 minutes at a heating block. At the same time, the electrophoresis box was filled with running buffer, after inserting the gel. After heating, samples were centrifuged for 1 minute before 30 µL of sample with loading buffer were loaded onto the gel. At least one lane per gel was loaded with 5 µL protein ladder. A voltage of 80 V was applied to the chamber until the bromophenol blue (from loading buffer) had reached the middle of the gel. The voltage was then raised to 150 V, and the gel was run until the bromophenol blue had reached the end of the gel. The gel was removed from the chamber and the gel, the membrane (after been activated in methanol for 25 seconds) and the filter papers were left in 1x transfer buffer for 10 minutes. Components were laid as a sandwich in the transfer chamber with following order, bottom to top; filter paper – membrane – gel – filter paper. A voltage of 8 V was applied to the chamber, and left overnight at 4 °C.

The following day the membrane was first blocked for 1 hour in 5 % non-fat milk in TBS-T solution at room temperature. Subsequently the membrane was transferred to a tube containing the appropriate primary antibody solution (see ***Table 1*** for antibody overview and ***section 5.2.2*** for information about dilutions and solutions used). No washing step was necessary between blocking and primary antibody incubation due to use of the same solution (milk). All incubations were performed using 14 or 50 mL tubes (based on membrane size) containing 3 or 6 mL antibody solution, respectively. The tube was left for reaction on a roller overnight at 4 °C. On the following day, the membrane was washed with PBS (3 x 5 min).

The membrane was transferred to a tube containing secondary antibody solution (***Table 2*** and ***section 5.2.2***) and incubated for 1 hour at room temperature on a roller**.** The secondary antibody was chosen based on the origin of the primary antibody (anti-rabbit, anti-goat or anti-mouse). The membrane was washed with TBS-T (3 x 5min).

Visualizing

To visualize the bands after the last washing step, the membrane was transferred to a transparency film. Equal amounts (0.5 mL per membrane) of the ECL liquids were mixed in a tube, and evenly applied to the membrane (1 mL combined liquid amount per membrane), which was left to react for 3-5 minutes in the dark, before covered with a new transparency film. When visualizing in the imaging system, chemiluminescence was chosen as setting. Exposure time was set based on antibody performance. For actin a short exposure of 5-20 seconds was chosen, while 1000–3000 seconds were used for CYP27a1.

5.4 Sample preparation prior to LC-MS/MS

5.4.1 Gel electrophoresis

Gel electrophoresis (GE) was performed to separate proteins according to size before further sample preparation. The same procedure used for WB, was used.

5.4.2 Protein digestion

Protein digestion was performed using two different approaches, in-solution and in–gel digestion based on the former separation step.

In-solution

The protein solution was diluted to 100 µL with 8 M urea /50 mM ABC. The proteins were reduced adding DTT to a final concentration of 5mM, mixing and incubating for 30 minutes at 37 °C. IAA was added to a final concentration of 15 mM to alkylate the protein chains, mixed and incubated in the dark at room temperature for 30 minutes. If urea was used to solve the proteins, 50 mM of ABC was added until the urea concentration was less than 1M. Trypsin was then added to a final trypsin + protein ratio of 1 + 19 (w/w) and left in a heating

block set to 37 °C with shaking, for at least 18 hours. To terminate the trypsin activity, TFA was added to a final concentration of 0.1 %. The tryptic digest was now ready for SPE.

In-gel

Following GE, the gel was covered by fixation buffer for 4-18 hours and stained with Coomassie brilliant blue for 2-4 hours. The gel was destained in water overnight. The mass range of interest (50kDa-100kDa for CYP27a1) was cut out from each lane, sliced into small pieces (1 mm^2) and transferred to a vial. 200 µL freshly made 25 mM ABC were added and allowed to be "swallowed" by the gel for 15 minutes. The supernatant was then discarded. 200 µL of ACN were added to the gel pieces and the supernatant was removed after 15 minutes. The two steps were repeated three more times. 200 µL of freshly thawed 10 mM DTT were added to the gel pieces and allowed to react for 30 minutes at 65 °C. Supernatant was removed and the two steps with ABC and ACN were repeated once. 200 µL of freshly thawed 10 mM IAM were added and the solution was left for 45 minutes in the dark at 37 °C. After removing the supernatant, the step with ABC and ACN was repeated once. 10-20 µL of 20 ng/µL trypsin/Lys-C mixture was added to the gel pieces and allowed to rehydrate for 10 minutes before adding 60 µL of 25 mM ABC. The gel pieces were stored overnight at 37 °C in the trypsin/Lys-C solution. The next day, 10 µL of 1 % FA were added before the supernatant was transferred to a protein low bind tube. 2 x 50 µL of 5 % FA + ACN (1 + 2) were added to the gel pieces and left for 15 minutes before the supernatant was combined with the other supernatant. Samples were dried in the concentrator and resolved in 1 mL 2 % ACN in 0.1 M TFA before SPE.

5.4.3 Off-line SPE

The SPE column was first activated with 1 mL 0.1 % TFA in ACN followed by 3 x 1 mL 2 % ACN in 0.1 % TFA. The sample was then added to the column and the flow-through was collected and passed through one more time. The column was washed with 0.1 % TFA in LC-MS water before eluting the sample with 1 mL ACN in 0.1 % TFA. After elution, the filtrate was dried in a concentrator on medium drying rate (no heat if dried overnight). Samples were then frozen at -80 °C until further use.

Before analysed by LC-MS, samples were thawed and diluted in 20 µL 2 % ACN in 0.1 % TFA and protein concentrations were measured. If protein concentrations were above 1

µg/µL, the sample was diluted to 1µg/µL in order to not inject more than 1 µg sample on the LC column.

5.5 LC-MS/MS

5.5.1 LC

1 µL of sample was injected (flow rate of 20 µL/min) using the auto sampler implemented in the nanoLC pump. The solvent gradient started at 3 % MP B and was increased to 36 % MP B during 30 minutes, followed by an increase to 95 % during 10 min and held at that percentage for 15 minutes. The gradient flow rate was 130 nL/min. For sample loading 5 µL of the loop-volume containing the 1 µL injected sample, was loaded to the precolumn with a flow rate of 500 nL/min. Before each sample the precolumn was equilibrated using 2 µL MP A and a flow rate of 500 nL/min, while the analytical column was equilibrated using 5 µL MP A and a flow rate of 200 nL/min.

5.5.2 MS

A voltage of 1.8 kV was applied to the nano emitter, and all experiments were performed in positive mode. Both full-MS with data dependent MS/MS (dMS/MS) and tMS/MS were used. In dMS/MS, resolution was set to 70 000, with automatic gain control (AGC) at 1 000 000, a maximum injection time (IT) of 120 ms and a scan range from *m/z* 350 to 1850. When turning to dMS/MS the resolution was lowered to 35 000, AGC target to 100 000 and IT to 60 ms. The normalized collision energy (NCE) was 30 eV, charges at 1 or >7 were excluded, and dynamic exclusion was set to 40 s. When tMS/MS was used, the *m/z* to the signature peptides were implemented to the PRM method, with an *m/z* isolation window of +/- 4. No retention time was set, but IT was set to 500 ms. Resolution was 35 000, AGC 100 000 and NCE 35 eV.

5.5.3 Data processing

The dMS/MS result files were evaluated using Proteome Discoverer where result files were search against CYP27a1 using Sequence Algorithm with the following criteria; maximum 2 allowed missed cleavages, precursor and fragment mass tolerance of 10 ppm and 0.6 Da respectively, no false discovery rate and with methionine oxidation as dynamic and

carbamidomethyl as static side chain modification. Signal to noise (S/N) threshold was set to 1.5 and minimum ion count to 1.

Both dMS/MS and tMS/MS results were evaluated in XCalibur software. Chromatographic peaks for targeted peptides were extracted with enabled boxcar smoothing (7 points) and a mass tolerance of 5 ppm. 5 decimals of the *m/z*-value were implemented.

6 Results and discussion

In this thesis a method for targeted CYP27a1 has been pursued. Beta-catenin was also included in the method investigations to evaluate the sample preparation workflow. An overview of the workflow is shown in ***Figure 15***.

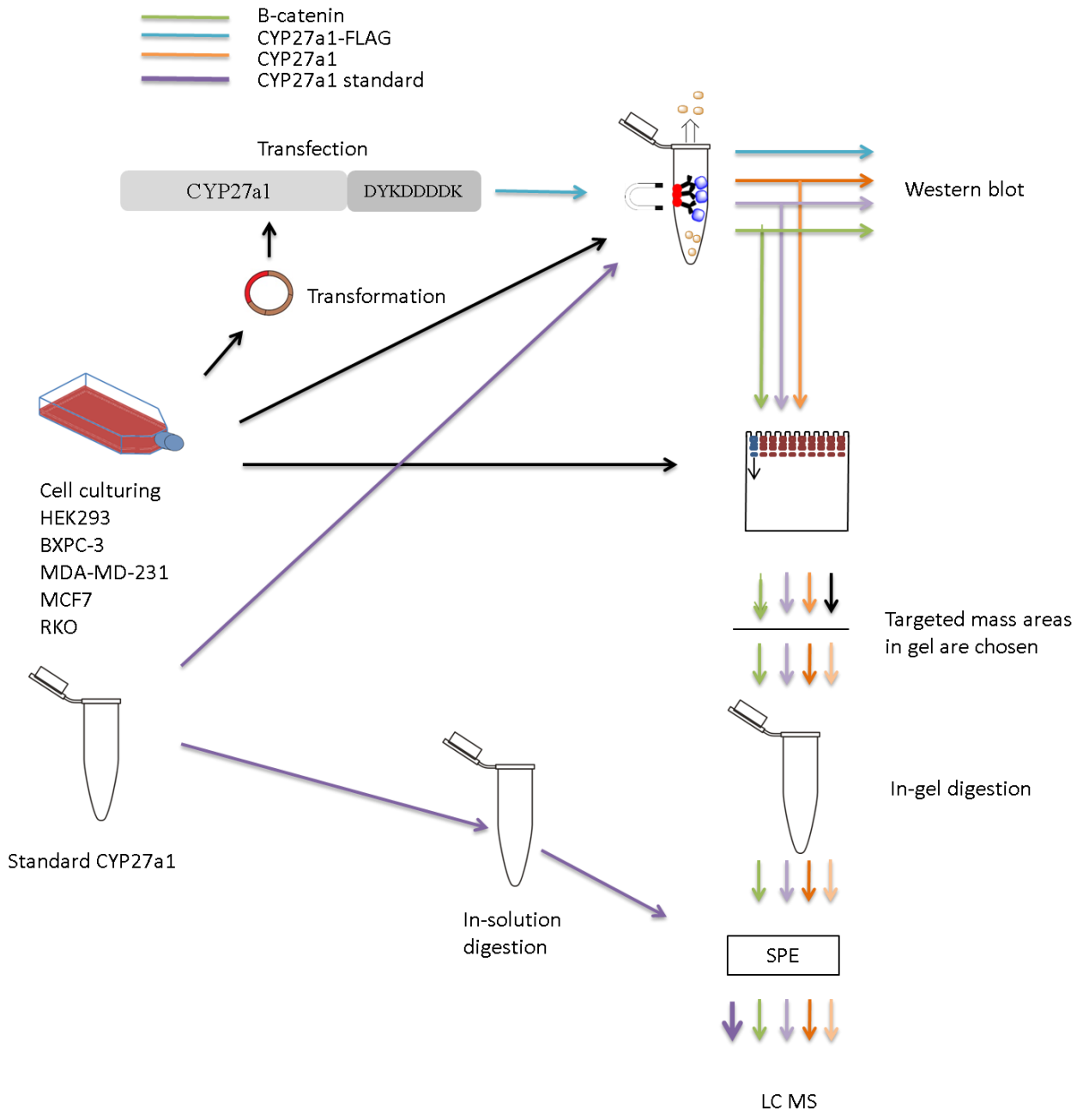


Figure 15 Overall workflow for the experiments performed in this thesis and discussed in the following section.

6.1 CYP27a1 – its abundance in different cell lines

The abundance of a protein differs in different cell types based on their origin (e.g. breasts) and disease (e.g. cancer). CYP27a1 levels in five cell lines were therefore determined by WB (***Figure 16A***). The CYP27a1 antibody used in WB was evaluated earlier by Kaja Lund [21]. Because band intensities for the loading control (actin) differed, Image lab was used to measure the intensities. By normalizing the actin/CYP27a1 intensity values to the average actin/CYP27a1 value in the cell line MCF7, a comparison of CYP27a1 levels could be made (***Figure 16B***). All WB raw files are found in ***section 9.3*** in appendix.

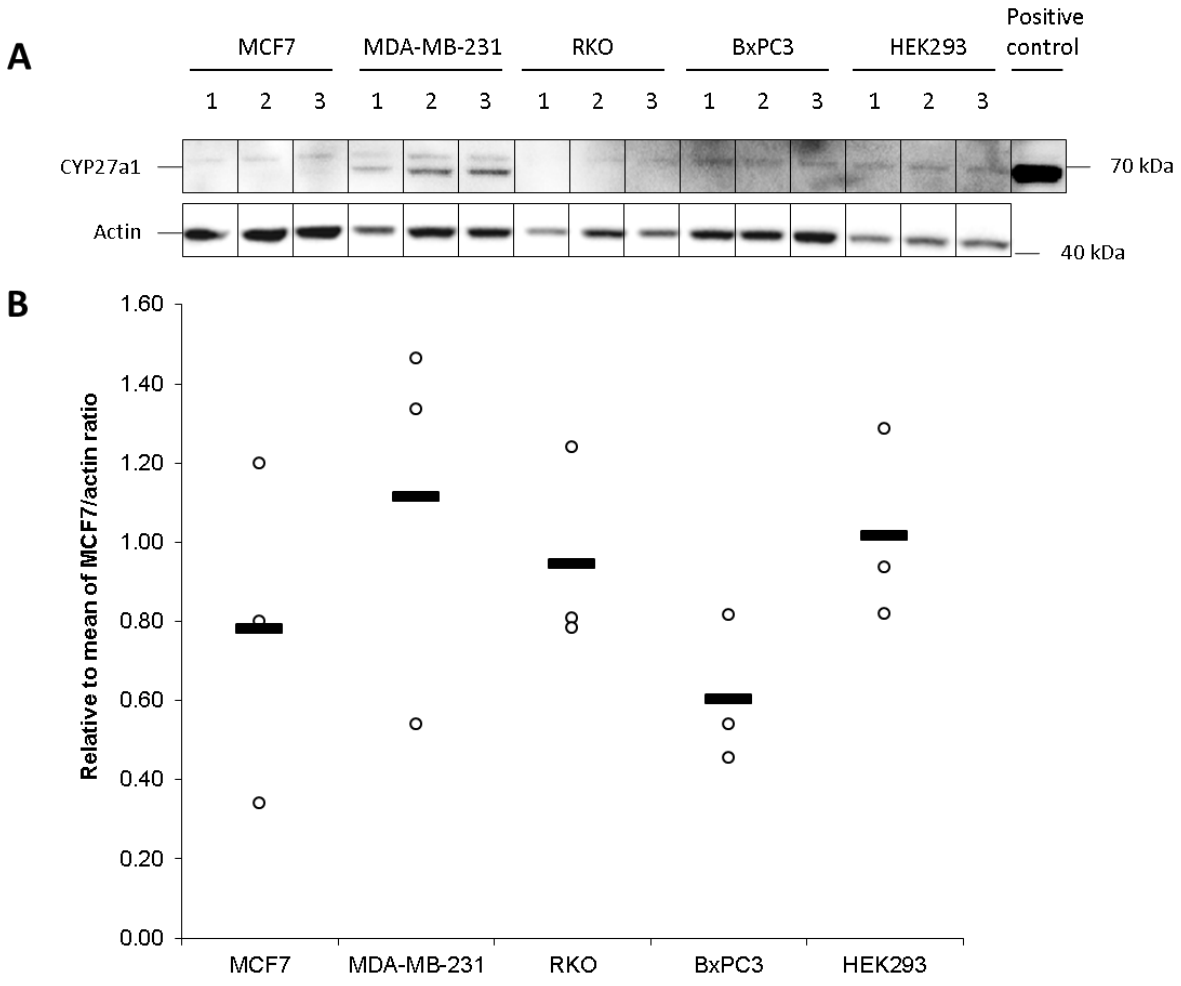


Figure 16A) WB of MCF-7, MDA-MB-231, RKO, BxPC-3 and HEK293 cell lines using CYP27a1 antibody. *Three replicates of each cell line were produced and analysed. For loading control actin antibody was used. As positive control, a transfected HEK293 cell line (CYP27a1-FLAG) and anti-FLAG antibody was used. B) The band intensity of CYP27a1 divided by the intensity of the corresponding actin band. This ratio is then divided by the MCF7/actin average. The black lines show the average of the values for the cell line. Both A and B are published in [89].*

The WB of the cell lines (**Figure 16*A***) only gave weak bands for CYP27a1, but it was possible to perform an approximate comparison of the CYP27a1 levels in the different cell lines. In MDA-MB-231, an additional band appeared close to the CYP27a1 band. It could be due to an unspecific binding of the antibody, or a modification of CYP27a1. As the second band was not at the same kDa as CYP27a1, the CYP27a1 intensity was measured based only on the upper band.

MDA-MB-231 was the cell line with highest amount of CYP27a1, and following cell work was performed using this cell line. In addition the HEK293 cell line was used, as it is easy to work with, e.g. regarding transfection. During cell experiments mycoplasma infection was an issue, and the cell culturing had to be repeated.

Thus, the cell lines MDA-MD-231 contained the highest amount of CYP27a1. Further investigations were performed using the MDA-MB-231 cell line, and the HEK293 cell line.

6.2 Producing a CYP27a1 standard

In the onset of this study a CYP27a1 protein standard was not available, and antibodies for CYP27a1 performed poorly. As the method development included investigation of possible sample treatments which partly relied on antibodies, transfection (***3.3.2***), where DNA for the CYP27a1-FLAG protein was implemented in the cells, was used to produce a CYP27a1 standard appropriate for immuno-based experiments. The antibody for the FLAG amino acid sequence was commonly used in the lab for selective detection by western blot, and was by that considered to provide the selectivity needed.

Fluorescence evaluation

The response of a cell regarding protein expression is not visible to the eye, and in order to quickly evaluate the success of transfection, EGFP was used. Thus, in one replicate EGFP-DNA was inserted instead of CYP27a1-DNA, functioning as a positive control. The EGFP emits fluorescent light, and ***Figure 17*** shows a picture of the EGFP-cells taken through a microscope.

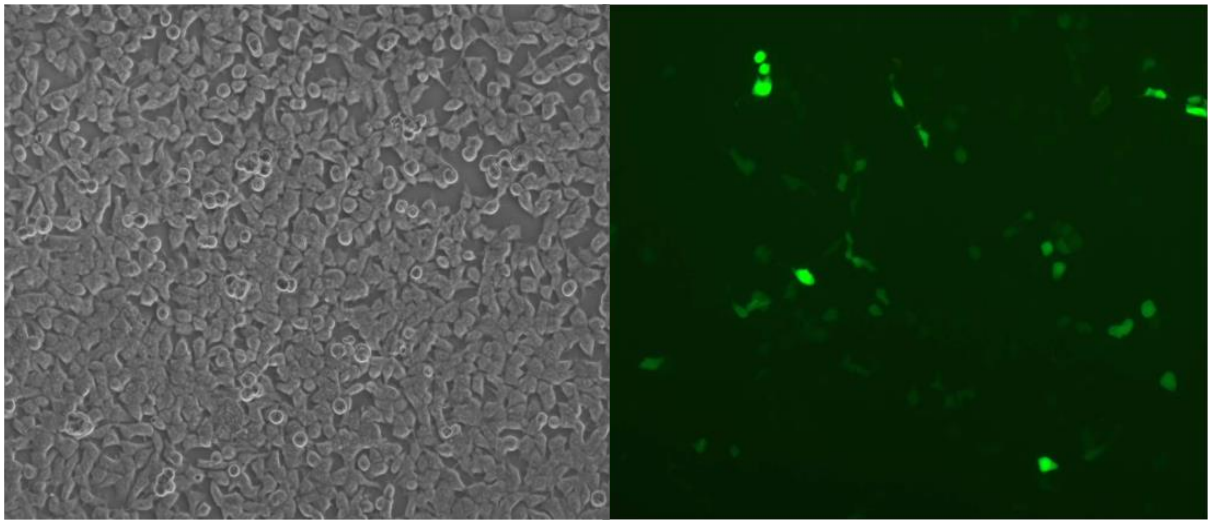


Figure 17 Microscope pictures of transfected HEK293 cell line with EGFP-DNA. *The picture to the left is with normal setting (shows the cell density). The picture to the right is taken in fluorescent mode (EGFP-proteins produced by transfection). A negative control was included in the experiment (data not shown).*

The amount of green dots among the cells implies that the transfection was successful, and that EGFP proteins were produced. Based on this it could be assumed that CYP27a1-FLAG proteins were produced in the other wells, where the CYP27a1-DNA was applied. However, as DNA is produced in bacterial cells and inserted in mammalian cells, each of those components are "alive" and prone to variable behavior and response. Hence, no definite conclusion regarding successful transfection could be made based on the successful EGFP-protein transfection, and further confirmation was wanted.

WB evaluation

To further evaluate the transfection WB was used, where a transfected cell lysate and an untreated cell lysate, both HEK293, were compared (***Figure 18***). By using anti-FLAG antibody, a band was expected for the transfected lysate, but not for the untreated one. In addition, WBs with CYP27a1 antibody were performed on the same samples in order to 1) compare the CYP27a1 antibody to the FLAG-antibody 2) to compare CYP27a1 levels in transfected and untreated cells and 3) use as a positive control (i.e. that CYP27a1 was present in both samples.

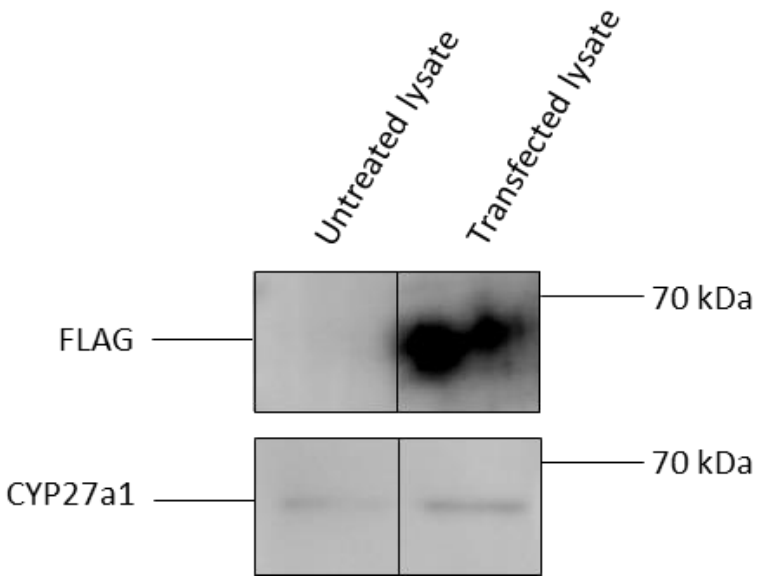


Figure 18 WB of untreated cell lysate and transfected lysate (CYP27a1-FLAG), using antibody for FLAG and CYP27a1. *The same transfected and untreated lysate was used for both antibodies, and the same protein amount was applied to the gels.*

For the FLAG-antibody, bands appeared only for the transfected lysate, and the intensity of the CYP27a1 bond for the transfected lysate was stronger than for the untreated lysate. Both observations corresponds to that CYP27a1-FLAG was produced. Based on this, CYP27a1 had been produced by transfection, and could now be used as standard in investigations of immuno-based experiments, providing enrichment (IP) and detection (WB). In WB, a sample containing the FLAG sequence could be applied, functioning as positive control (detected by the FLAG antibody) when relaying on the CYP27a1 antibody for detection.

In addition, assuming that the same amount of protein was applied to the gels, the intensity of the bonds can be used to evaluate the antibody performance. For the transfected sample, the FLAG band was significantly stronger than the CYP27a1 band. As the CYP27a1 antibody detects both CYP27a1 and CYP27a1 FLAG, while FLAG only detects CYP27a1 FLAG produced by transfection, the intensities should have been opposite. This demonstrates the other issue with the CYP27a1 antibody; its low sensitivity. However, to be able to conclude regarding the sensitivity, actin control should be included as the actin protein is used as a loading control in WB. The low increase in intensity in the transfected lysate CYP27a1 band, compared to the CYP27a1 band in the untreated lysate could imply an uneven protein application to the gel.

Thus, CYP27a1-FLAG was produced by transfection and could be used further for immuno-based experiments.

6.3 Immunoprecipitation

As the CYP27a1 abundance is low in cells, and the samples are complex, sample clean-up was necessary prior to the LC-MS determination. IP (***Figure 14***) was used for this purpose as it can provide both decreased sample complexity and an enrichment of the target compound. A workflow for all IP experiments performed is found in ***Figure 19***. During IP investigations, a method for LC-MS determination of CYP27a1 was not established (no CYP27a1 standard available), and WB was used for protein detection and assessment of the IP experiments.

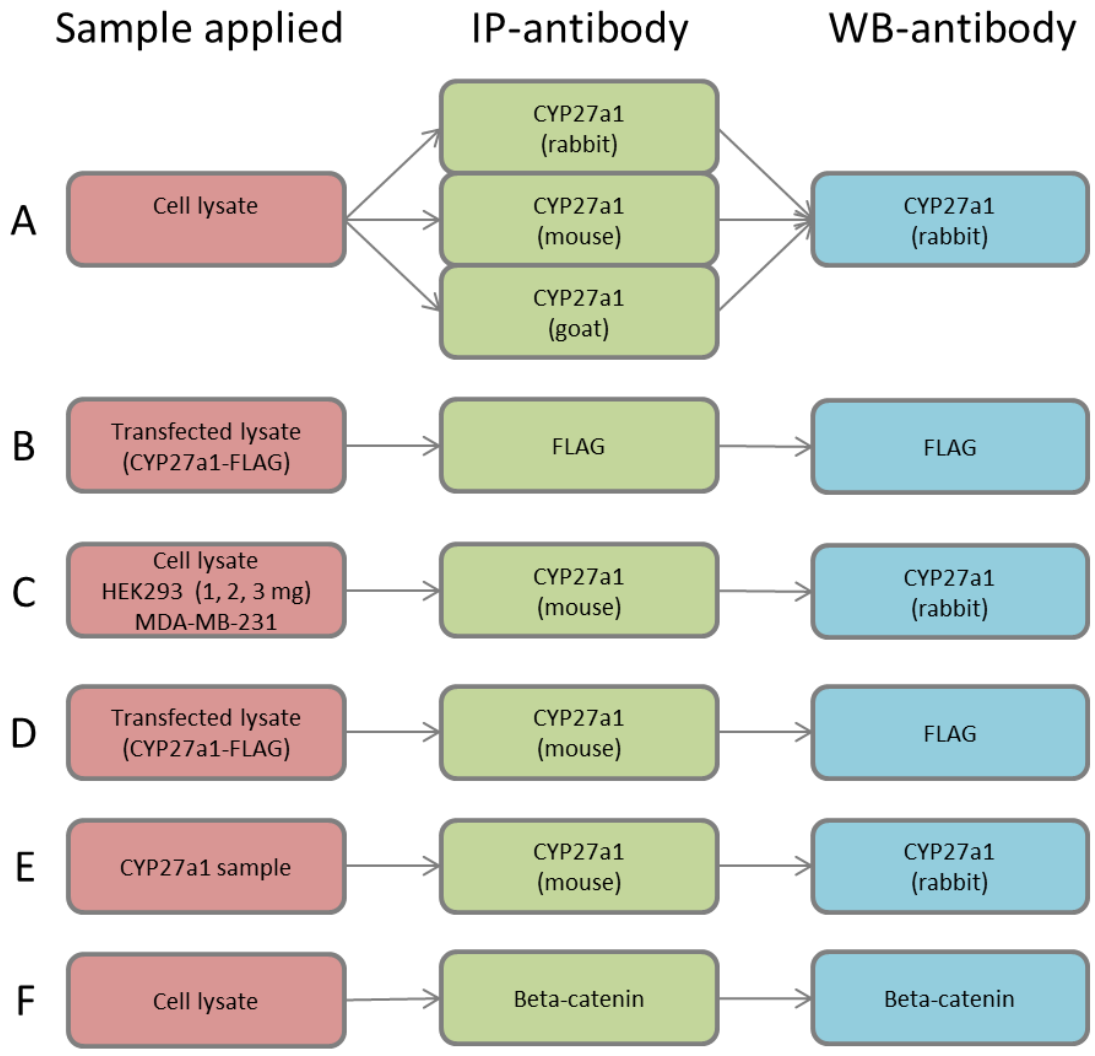


Figure 19 Experiments performed to investigate IP. *The main difference is antibodies used (to use FLAG antibody a transfected lysate must be applied, as CYP27a1 in normal lysate does not contain the FLAG amino acid sequence). If not mentioned otherwise in the figure, the HEK293 cell line is used. The antibody origin is mentioned for CYP27a1, to be able to differ between the three different CYP27a1 antibodies used in this thesis.*

IP was used to target CYP27a1 using CYP27a1 antibodies. Three different antibodies were evaluated (***Figure 19A***) from three different origins and suppliers. In ***Figure 20*** the WB of the IP sample in which the CYP2C7a1 goat antibody was used, is shown. This antibody was the only one that appeared to give an extraction of CYP27a1, as an indication of a band is seen for CYP27a1 in both the cell lysate and the IP sample, but not in the negative control. WB signal was poor, especially as the CYP27a1 band in cell lysate normally was more intense. However, better signal could not be obtained, and it was decided to use the CYP27a1 goat antibody in the future experiments.

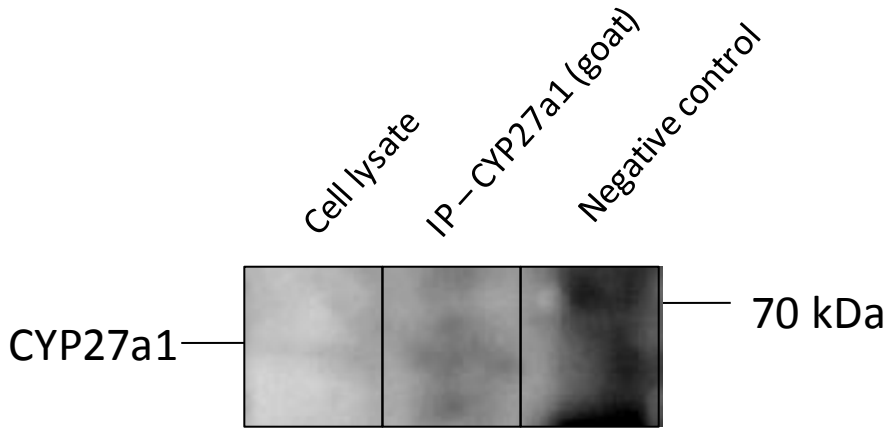


Figure 20 WP of IP using CYP27a1 goat antibody, with positive control (cell lysate) and negative control.

Thus, the CYP27a1 goat antibody was evaluated to be the best choice for further IP extraction of CYP27a1.

IP targeting FLAG

As the IP-WB workflow for CYP27a1 gave low signal intensities, FLAG antibody was used (***Figure 19B***), targeting CYP27a1-FLAG in the transfected cell lysates, to evaluate the general IP procedure. In ***Figure 21*** the WB of an IP-FLAG sample is compared with that of a transfected only sample (i.e. not performed IP) using FLAG antibody.

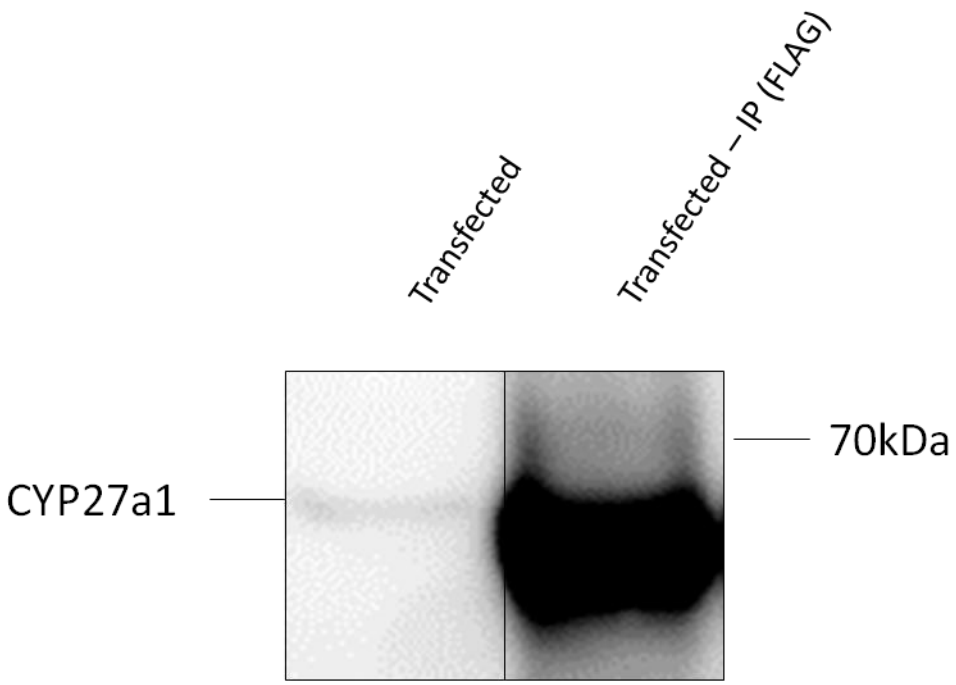


Figure 21 WB with IP-FLAG detection. *The band on the left is transfected lysate, and the band on the right is IP FLAG antibody treated transfected lysate. The FLAG antibody was used for detection for both samples.*

The WB showed that the IP method enabled the extraction of a CYP27a1 using a FLAG antibody. The minimal/no WB detection for former attempts of CYP27a1 IP were then most likely due to the antibody performance and not the method itself or the operator. The enhanced intensity of the IP band compared to the no-IP band indicates that the CYP27a1 concentration is increased after performing IP. However, loading control with actin could not be done as the only protein present in IP samples should be the targeted protein, and the enrichment effect of IP was therefore an estimate based on the applied protein concentration.

Thus, targeting FLAG in IP enabled enrichment of CYP27a1-FLAG.

Further IP investigations

A closer investigation of the IP procedure to find the limiting step regarding CYP27a1 extraction was needed. To see if the cell line used had an impact on the IP outcome, both HEK293 and MDA-MB-293 were evaluated (***Figure 19C***). As the amount of protein could affect the ability of extraction, three different amounts of protein were used for HEK293 (1 mg, 2 mg and 3 mg) (***Figure 19C***). In case that WB detection did not occur due to the performance of the CYP27a1 antibody in WB, transfected HEK293 sample was also subjected to IP (***Figure 19D***). With this sample, CYP27a1 antibody could be used in IP, while

the FLAG antibody could be used in WB, eliminating the use of a poor antibody in both steps. As a last test, the CYP27a1 standard (now available) was also subjected to IP (***Figure 19E***). A workflow for these experiments is found in ***Figure 22A.*** The WB results for the workflow are found in ***Figure 22B-E***.

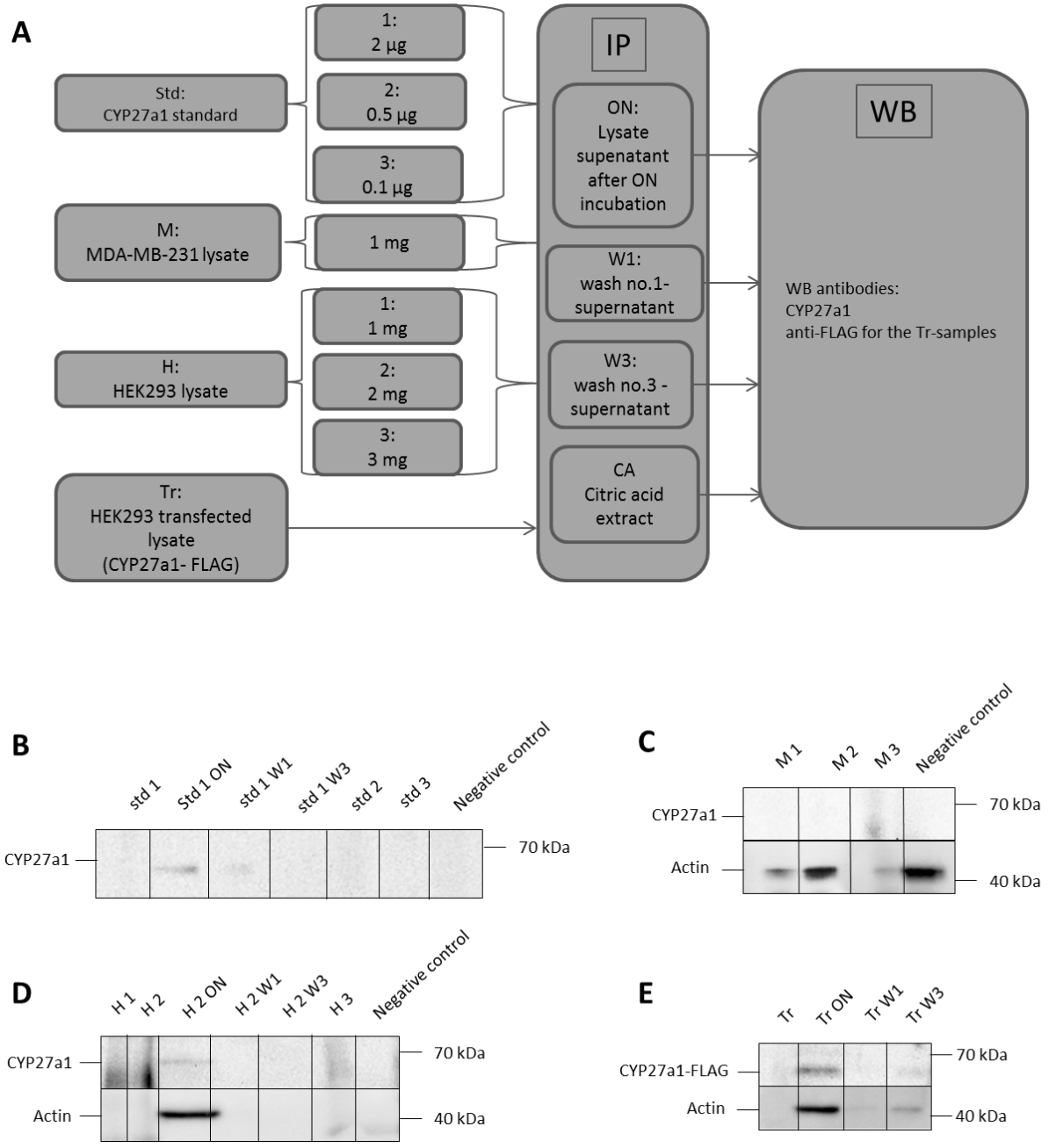


Figure 22A) Workflow for IP experiments. B) WB for standard CYP27a1, C) WB for MDA-MB-231, D) WB for HEK293 and E) WB for transfected HEK293. *Abbreviations: Std: CYP27a1 standard, M: MDA-MB-231 lysate, H: HEK293 lysate, Tr: HEK293 transfected lysate (CYP27a1-FLAG), ON: overnight supernatant, W1: wash no.1 supernatant, W2: wash nr.3 supernatant, CA: citric acid extract.*

For each sample four outputs were produced; to test that if the proteins did not attach to the bead-antibody complex, the supernatant after overnight incubation was collected (ON). Three washes were performed of each sample, and to see if this affected the bead-antibody-protein complex, the supernatant from the first (W1) and the third (W3) wash were collected. The last was the citric acid extract (CA) where it was expected to find CYP27a1. All of the IP experiments showed the same; no band occurred in CA, but band occurred in ON. These observations can be explained by either that the antibody binds to the protein, but not to the magnetic bead, or that the antibody binds to the magnetic bead, but not to the target protein. A hint of a band was observed for W3 in ***Figure 22E*** (transfected sample) and W1 in ***Figure 22B*** (CYP27a1 standard), but the appearance of a band for actin in the same samples indicated that this was ON that had not been washed yet (and not the loss of CYP27a1 from a possible bead-antibody-protein complex).

To conclude; adding more proteins or changing the cell line had no influence on CYP27a1 extraction, and neither did the amount of washing or the WB antibody used.

6.4 How to connect biology and chemistry for analysis of biologically prepared samples?

When IP samples were subjected to LC-MS analysis without other additional sample preparations steps than digestion (in solution) and SPE, CYP27a1 could not be detected, partly caused by detergents that gave rise to ion suppression in the electrospray.

At that time, the SDS loading buffer was used to elute proteins from the beads. When analysing biological samples by LC-MS, the use of detergents should be minimalized, due to their mismatch with ESI [90-93] . Hence, citric acid, a non-detergent and weaker eluting agent, was assessed for elution of proteins from the bead-antibody-protein complex in IP. Due to problems with the MS-instrument during IP investigations, the citric acid samples were not analysed by LC-MS, and hence, the detergent issues in these samples are not known. However, as the NP-40 detergent was still used for cell lysis, it was assessed as necessary to perform GE with subsequent in-gel digestion to achieve adequate MS detection.

With the use of SDS-PAGE detergents were not were no longer present in the spectra. The SDS-PAGE sample preparation with subsequent in-gel digestion is time consuming, and loss

of proteins is an issue [94], but it provides separations of proteins, enabling the extraction of protein in a selected mass range, also minimizing sample complexity.

Detergents are powerful agents for solubilization of a cell, most likely necessary for the extraction of the mitochondrial CYP27a1. Thus removal of detergents in the lysis buffer was not attempted at this stage of the investigations. An optimization of the workflow, including the use of non-detergent lysis buffers and hence possibly remove the need for SDS-page (if it is not necessary for minimization of sample complexity), should be performed.

It is not only proteins that contributes to the complex nature of cell samples, but also other cell components (cell membrane, metabolites), salts and particles e.g. from gel separation, could give rise to technical issues during an LC-MS analysis. Such issues are clogging (nano LC is especially exposed to clogging due to the small inner diameters) and ion suppression (due to salts or other easily ionisable compounds of high abundance). SPE was therefore performed on all samples prior to LC-MS analysis. The LC system contained a precolumn, but as clogging of precolumns occurred during the investigations, an additional off-line SPE step was assessed as necessary. In further investigation, the use of an automated filtration/filter backflush (AFFL)-SPE system [95] could remove the need for the time-consuming off-line SPE stage.

Thus, it was assessed as necessary to perform SDS-PAGE with subsequent in-gel digestion to remove detergents, and SPE for additional sample-clean up before LC-MS/MS analysis.

6.5 Beta-catenin; does the IP method work for other targets?

The investigated method for detection of CYP27a1 in cell samples by IP was not successful for identification of CYP27a1. It was suspected that the reason was unsuitable antibodies; therefore it was decided to test the method by targeting beta-catenin. IP using beta-catenin (see ***Table (AP) 4*** for protein sequence), followed by WB (***Figure 19F***) was already used by co-workers, and in addition, determination of beta-catenin by LC-MS had been established in a previous study [96]. Beta-catenin was therefore targeted to verify the IP workflow in this study, and WBs of beta-catenin IP samples are found in ***Figure 23.***

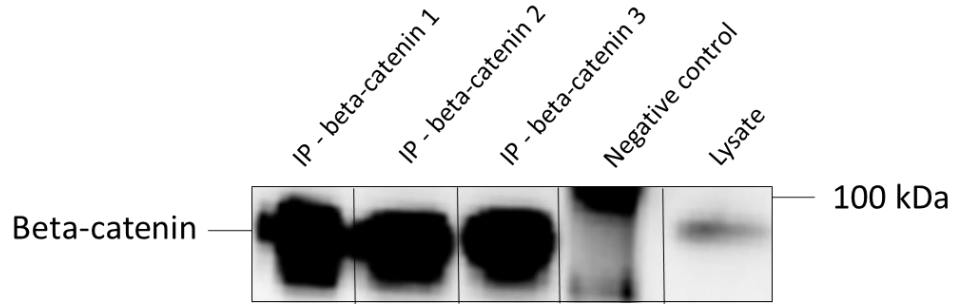


Figure 23 WB of beta-catenin IP samples. *Antibody against beta-catenin was used for all WBs. The IP-beta-catenin bonds represent three replicates of beta-catenin IP samples, where beta-catenin antibody was also used in IP. The lysate is an untreated cell sample, while normal IgG rabbit antibody was used as negative control in IP.*

Beta-catenin was detected by WB in IP samples, and thus the procedure used in IP was verified. The beta-catenin band intensity for the lysate sample, which is untreated, was much weaker than for the IP bands, supporting the trait of IP as an enrichment step. No band appeared in the negative control at the beta-catenin mass range.

Bands detected by WB cannot be further analysed by LC-MS, hence, before LC-MS analysis, proteins in the IP samples were separated using SDS-PAGE (***3.3.4***). From the stained gel three lanes were combined to one sample to enhance beta-catenin abundance before in-gel digestion. The stained gel and MS chromatograms are shown in ***Figure 24***.

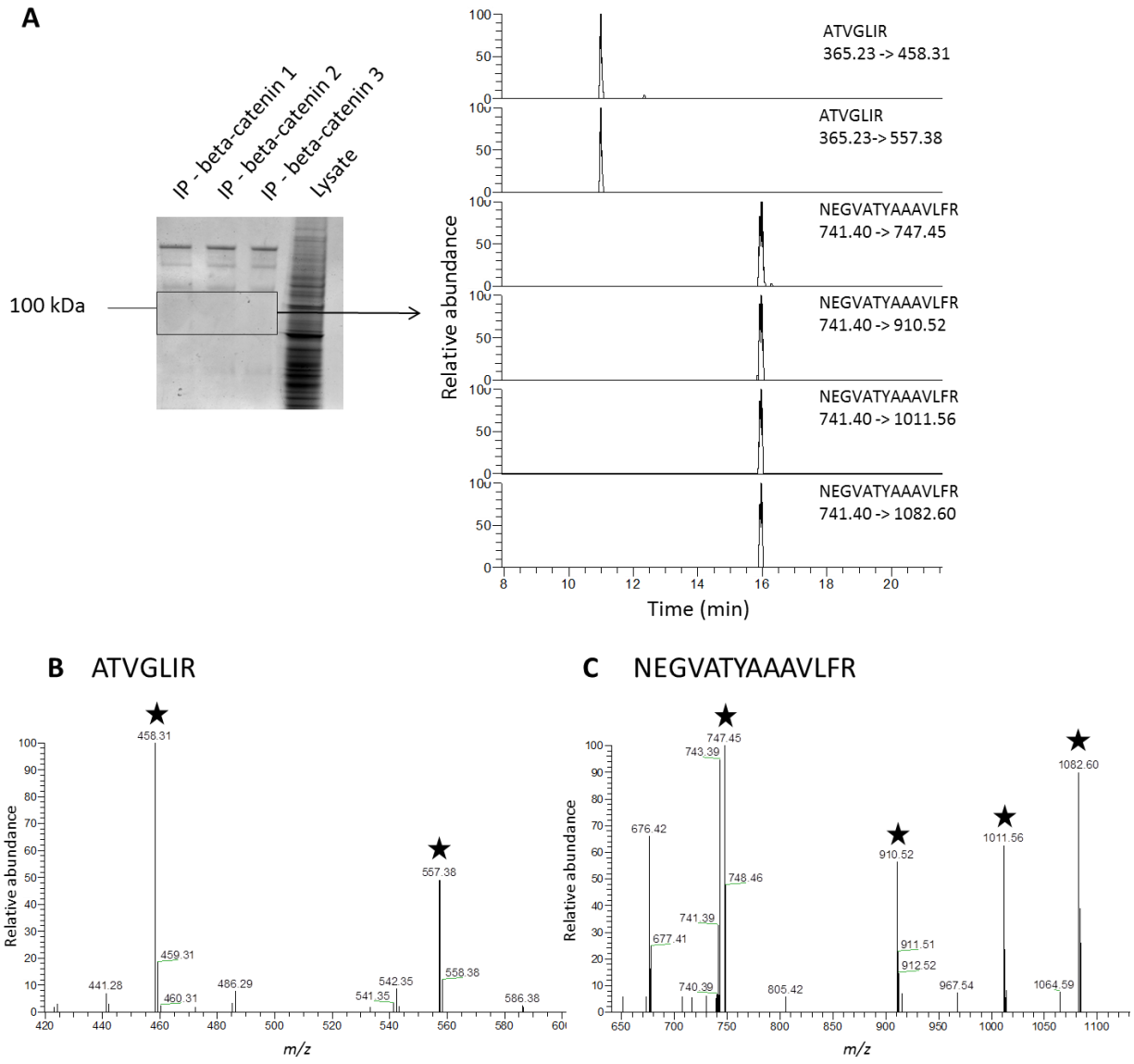


Figure 24 Gel bands and extracted ion chromatograms for the two beta-catenin signature peptides, ATVGLIR and NEGVATYAAAVLFR. *The precursor and fragment ions for each peak are listed. B) The extracted mass spectra for the retention time of ATVGLIR. C) The extracted mass spectra for the retention time of ATVGLIR. The stars indicate the fragment ion m/z peaks. The in-house packed silica monolithic C8 precolumn (5 cm x 50 µm) and the in-house packed C18 column (15 cm x 50 µm) were used. Flow rate was 130 nL/min, with a gradient of 3-36 % MP B. The PRM method targeting beta-catenin was used, with tMS/MS settings described in the experimental section.*

In the stained gel, beta-catenin was not detected by Comassie blue. New IP samples were produced to look for the bands, but they did still not occur. However, this is not an issue as Comassie blue staining is not sensitive and according to WB, a more sensitive method with the use of the beta-catenin antibody, beta-catenin was present. The additional bands occurring in the stained gel in ***Figure 24*** also occurred to some extend in the WB (see the WB raw file

in ***Figure (AP) 9*** in appendix), but it was not investigated further as the bands were located at other masses.

Beta-catenin was successfully detected in IP samples by nano LC-MS. An overview of the now established method for IP LC-MS is shown in ***Figure 25***. The sample was injected 5 following times on the nano LC-MS system, applying PRM. Signature peptides with corresponding transitions for beta-catenin [96] are listed in ***Table (AP) 6*** in appendix. The replicates showed a high repeatability in retention time (ATVGLIR; mean = 10.91, standard deviation (s) = 0.01, relative standard deviation (RSD) = 0.05, NEGVATYAAAVLFR; mean = 15.88, s = 0.02, RSD = 0.13, N=5 for both peptides). However, the detection was only based on two of four listed signature peptides, as two on the peptides were not detected. It is not known why no peaks could be extracted, but this was not investigated further as an adequate determination could be based on two peptides identified [49]. For ATVGLIR only two fragments were determined, but again, with four fragments for NEGVATYAAAVLFR, data were considered sufficient for the purpose of the experiment; detection of beta-catenin in IP samples.

The repeatability of the WB of beta-catenin IP was to a certain degree verified, as it was performed twice. Only one sample was analysed by LC-MS, but five LC-MS replicates provide sufficient data to verify the LC-MS method for the LC-MS system used. The repeatability of the entire method was not evaluated, but the amount of data obtained was enough for the purpose of this investigation.

*To sum up, beta-catenin was detected in IP gel samples by targeted LC-MS, hence; sample preparation procedure including IP (**Figure 25**) enables detection of a target protein by LC-MS.*

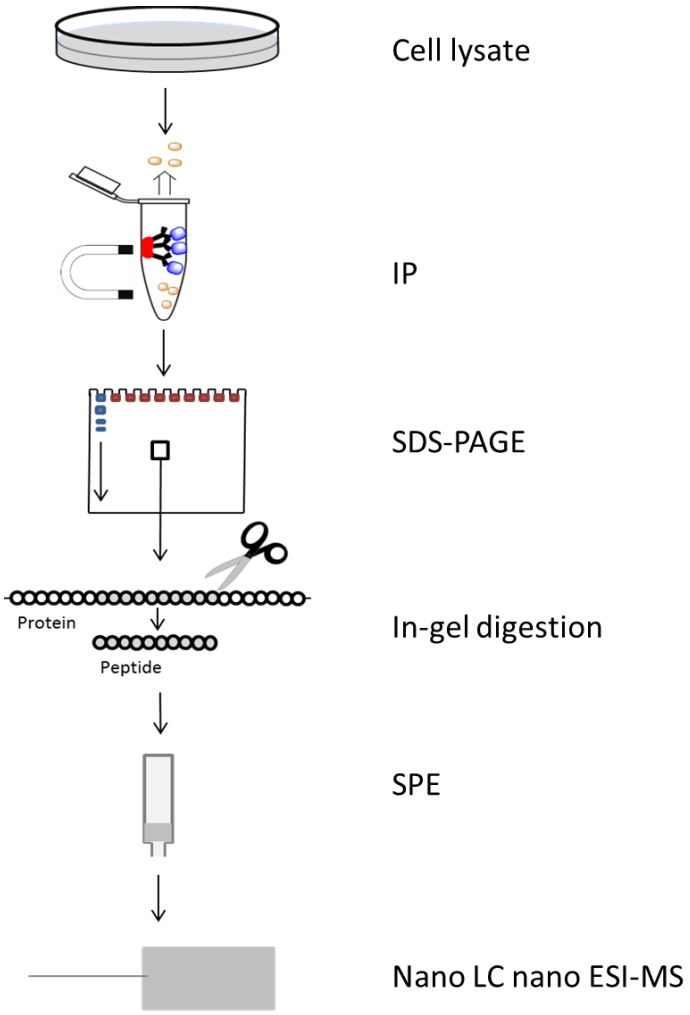


Figure 25 Established workflow using IP, for detection of beta-catenin.

6.6 LC-MS data for CYP27a1

6.6.1 Signature peptide determination

When applying PRM for targeted proteomics, the signature peptides corresponding to the *m/z*-values monitored in the MS must be determined.

A list over CYP27a1 peptides predicted as highly observable was found in the PeptideAtlas database [53, 54]. The listed peptides from PeptideAtlas were evaluated regarding uniqueness by Uniprot. This computer program matches the amino acid sequence against the human genome, and enables us to easy find if the peptide is matched to other proteins. If a peptide matched 100 % against other proteins than CYP27a1 it was discarded for further evaluation,

as it cannot provide a selective detection. The peptides matching e.g. 57 % to another protein was not excluded, but before choosing the peptide as signature peptide, the sequence overlap had to be investigated further. Investigations for ATGAPGAGPGVR and SIPEDTVTFVR are shown in ***Table (AP) 8***. The sequences did not give the same mass as the signature peptide candidates; hence they would not give rise to interferences in tMS/MS, and the signature peptides could be used for CYP27a1 detection.

An in-solution digested CYP27a1 standard was used for investigation of the practical assets of the peptides; chromatographic peaks and number of fragment ions detected. The result of the peptide evaluation described is shown in ***Table (AP) 7*** in appendix**.**

Finally a gel sample was analysed by LC-MS (in dMS/MS mode). For this sample, only LLKPAEAALYTDAFNEVIDDFMTR was detected with a good S/N ratio, and it was therefore decided to continue with this peptide as a signature peptide, even though it was not detected in the standard, and it contained methionine (oxidation).

Determination of a protein can be based on an single signature peptide, but to ensure a secure identification [49] three signature peptides were wanted in this study. Based on the evaluation described in the previously sections (a workflow overview is found in ***Figure 26***), the two additional peptides ATGAPGAGPGVR and SIPEDTVTFVR were chosen.

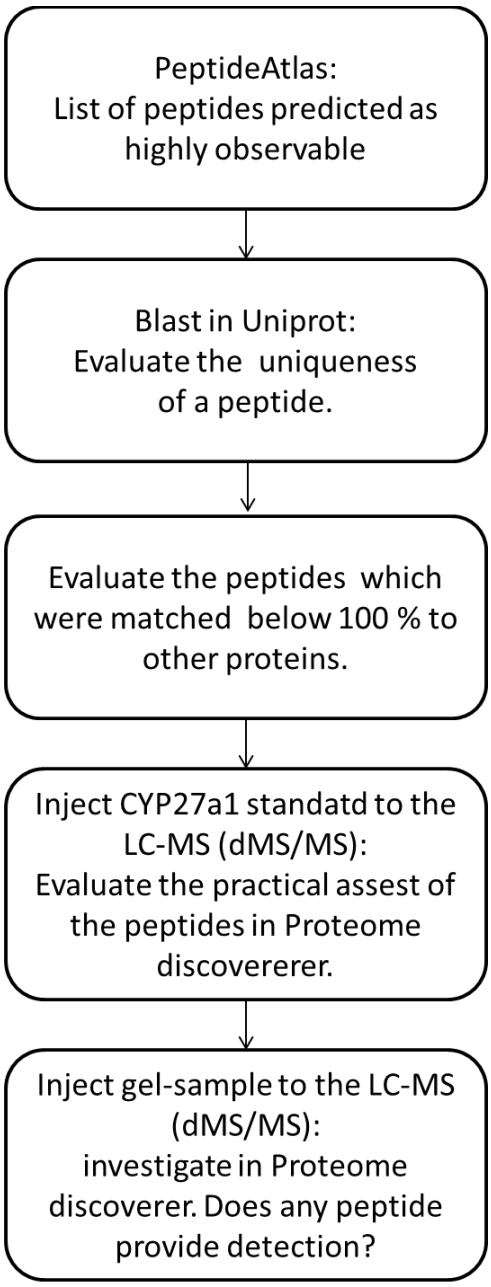


Figure 26 The workflow for choosing signature peptides. *All LS-MS evaluations were performed in dMS/MS mode.*

Identification of a peptide, and hence the CYP27a1 protein, is usually based on at least three fragment ions [97]. To decide which fragment ions to use to evaluate identification, a tMS/MS analysis by PRM of the CYP27a1 standard was performed, implementing the three signature peptides into the PRM method. The chromatographic peaks corresponding to the y-fragment *m/z*-values for ATGAPGAGPGVR and SIPEDTVTFVR, with mass spectra, are shown in ***Figure 27.***

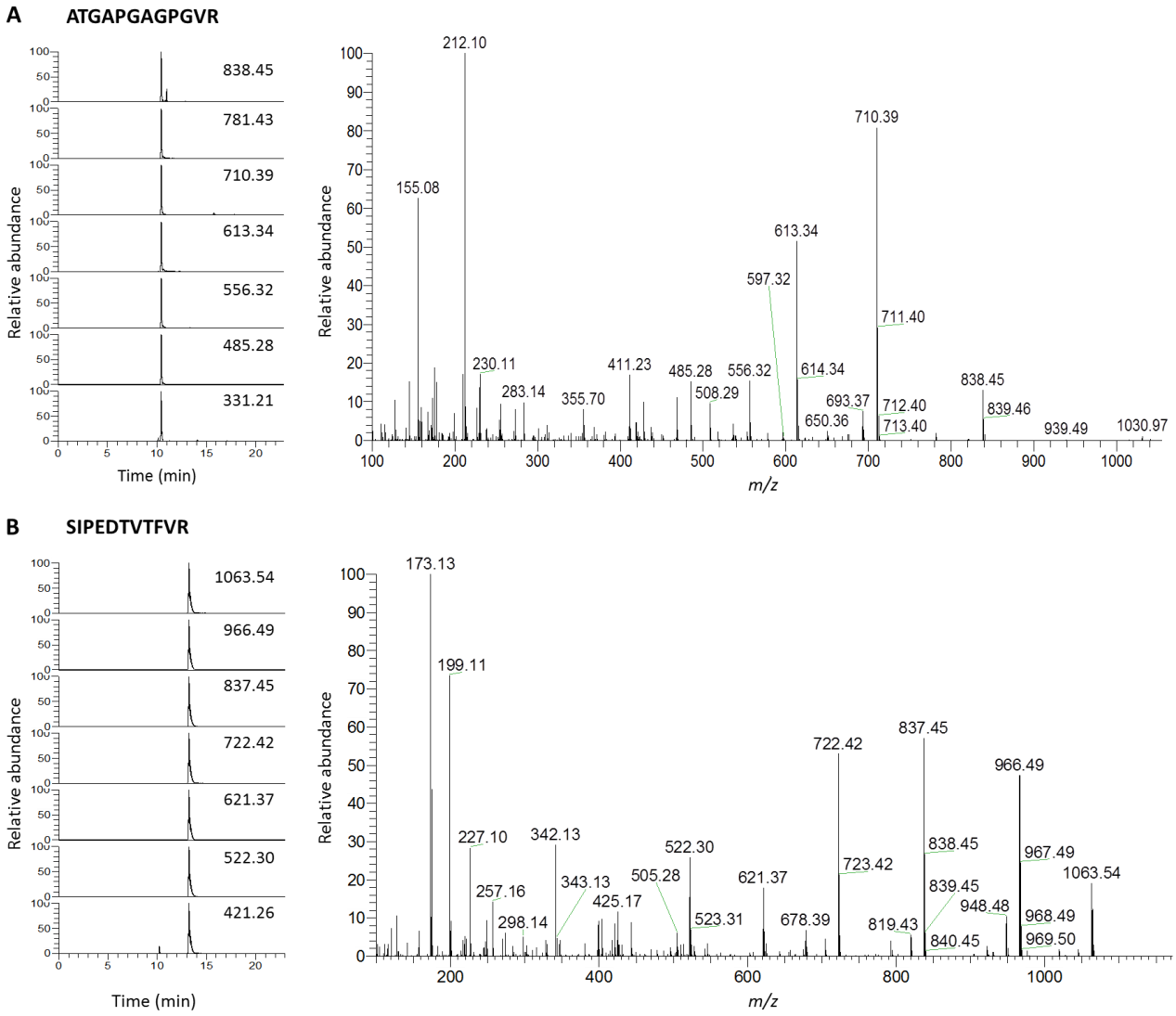


Figure 27A) Extracted fragment ion chromatograms with mass spectra for ATGAPGAGPGVR. B) Extracted fragment ion chromatograms with mass spectra for SIPEDTVTFVR. *In the chromatograms in A and B, the m/z-values to the extracted peaks are listed for each chromatogram. To the right, the mass spectrum for the chromatographic peak retention time is extracted. All injected replicates were analysed by tMS/MS mode. Chromatographic conditions and the analytical column used were the same as described in* ***Figure 24****. The commercial C18 Acclaim precolumn was used.*

LLKPAEAALYTDAFNEVIDDFMTR was not detected, and a fragment selection for this peptide has not been performed. For ATGAPGAGPGVR and SIPEDTVTFVR all implemented fragments gave rise to a chromatographic peak. The peaks for SIPEDTVTFVR in ***Figure 27B*** had some tailing. For each of the two peptides, the three fragments with highest intensity were selected for further use [97]. However for SIPEDTVTFVR, two of the three most intense fragment mass spectra peaks (*m/z* = 837.45 and 966.49), later showed to not give detection when analysing real samples. Therefore, in addition to the *m/z* = 722.42

fragment, *m/z* = 621.37 and 522.30 were chosen for SIPEDTVTFV. The three signature peptides are listed in ***Table 5***, with fragments for SIPEDTVTFVR and ATGAPGAGPGVR.

Table 5 List of signature peptides, with fragments, chosen for CYP27a1. *Three signature peptides were chosen based on a list of criteria, with three corresponding fragments. LLKPAEAALYTDAFNEVIDDFMTR did not provide fragment selection.*

CYP27a1 Peptide	Precursor $[M+2H]^{2+}$ *m/z*	Fragment *m/z*		
ATGAPGAGPGVR	505.77	Y_8^+ 710.39	Y_7^+ 613.34	Y_6^+ 556.32
SIPEDTVTFVR	632.34	Y_6^+ 722.42	Y_5^+ 621.37	Y_4^+ 522.30
LLKPAEAALYTDAFNEVIDDFMTR	915.13	Not found		

Even though fragments were not chosen for LLKPAEAALYTDAFNEVIDDFMTR, the precursor mass was still implemented in the PRM method. Since the MS was set to only monitor three precursor masses, it was assessed that it would not affect the MS detection of the two other peptides. Identification of CYP27a1 was from now mainly based on the two signature peptides ATGAPGAGPGVR and SIPEDTVTFVR, which is still adequate for a secure identification (though more peptides would provide an even more secure identification [49]). The CYP27a1 protein with its signature peptides is illustrated in ***Figure 28.***

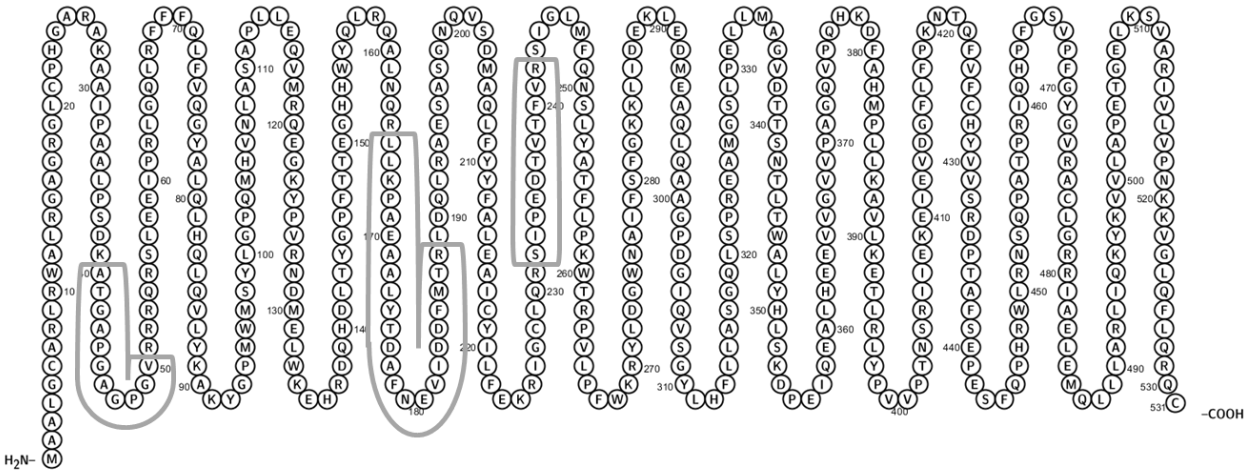


Figure 28 The CYP27a1 protein with signature peptides marked. *The three signature peptides are marked with grey. The protein is visualized using Protter [98]*

The signature peptide determination was time consuming and involved several evaluation stages, compared to using signature peptides suggested by databases available. Three signature peptides has also been established for an LC-M method for CYP27a1 in retina [72]. It was still assessed as necessary due to the instability of the system, the use of cells instead of retina, and the insecurity of the sample preparation.

To sum up; the two peptides ATGAPGAGPGVR and SIPEDTVTFVR enable a selective detection of CYP27a1, and could be implemented in the further investigations.

6.6.2 The liquid chromatography system

Precolumn evaluation

Precolumns have different ability to trap the analyte on the column, preventing the analyte to be flushed to waste when sample is loaded. Three different precolumns were evaluated regarding their performance; one commercial and two produced in-house. A benefit with using in-house produced columns is the cost reduction, as commercial columns often are expensive. The CYP27a1 standard was analysed in triplicate by LC-MS for each precolumn. The MS was set to targeted mode, scanning for the three signature peptides. In ***Figure 29***, the CYP27a1 peptide fragments are extracted for each precolumn tested.

In ***Figure 29A*** the commercial C18 Acclaim precolumn was used, coupled to the analytical system, and it provided detection of all six fragments. The peak tailing for the

SIPEDTVTFVR peptide (three bottom lanes) is consistent with previous observations. The in-house produced PS-DVB 5 cm precolumn (***Figure 29B***) did not give any chromatographic peaks, and hence no detection. This column was not evaluated further. The silica-based C8 monolithic column produced in-house [86] provided chromatographic peaks for all six fragments (***Figure 29C***). In the second and third chromatogram two additional peaks occurred but this did not affect the detection as the peaks were appearing with other retention times. The only effect of the additional peaks is the low relative appearance of the targeted peak in the second chromatogram (as the y-axis shows relative and not absolute abundance), but the intensity was the same as the other peaks (10^3-10^4). The tailing for the SIPEDTVTFVR peptide was also observed for this precolumn.

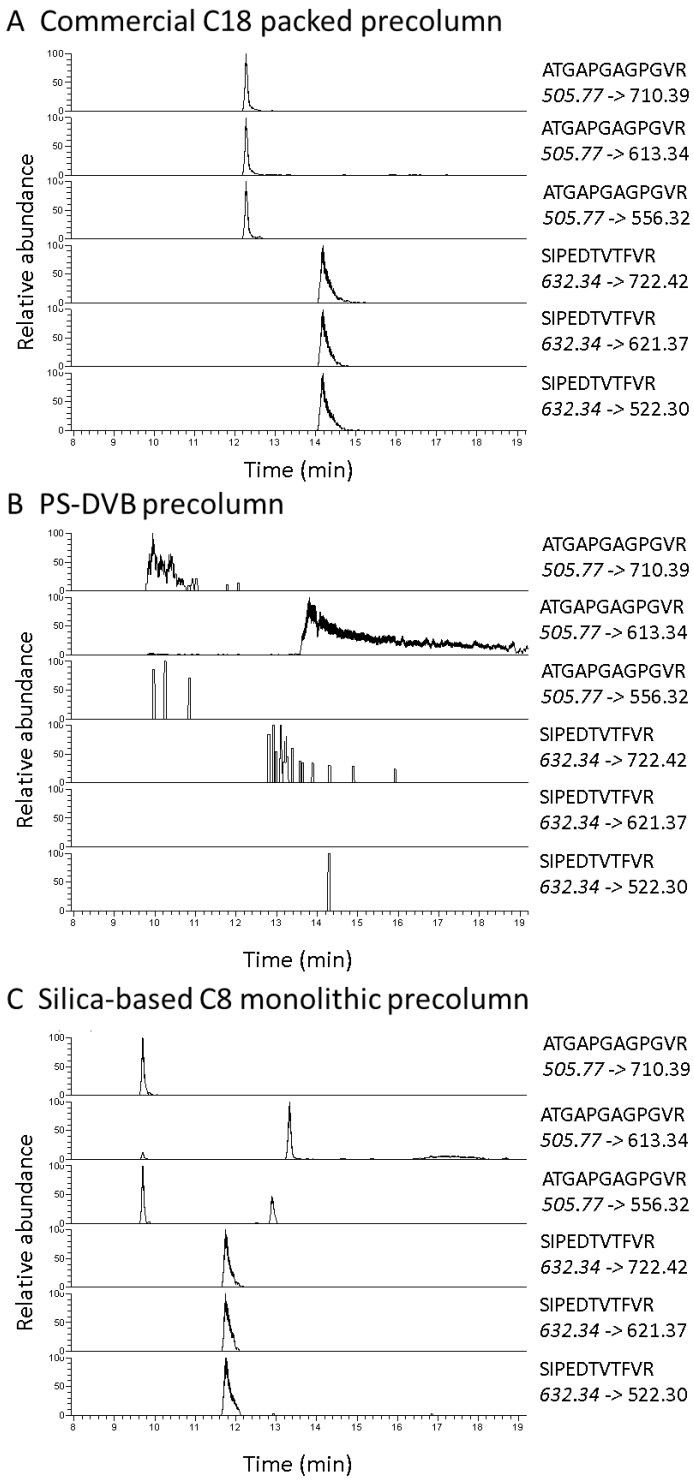


Figure 29 Extracted ion chromatograms of CYP27a1 peptides in a CYP27a1 standard analysed by three different precolumn LC-MS systems. *For each precolumn, the three fragment ions for the two signature peptides were extracted. The PRM method for CYP27a1 was used. With exception of the precolumns, the chromatographical conditions and analytical column used were the same as in* ***Figure 24.***

To more closely compare the commercial precolumn with the silica-based monolithic column, following parameters were assessed: stability in retention time, peak width, peak area and

peak intensity. The results of this investigation are illustrated in the plots in ***Figure 30***. The retention times were stable for three injections for both precolumns, but the retention time was shorter for the monolith column. A short retention time, without compromising the separation and detection is preferable. The peak width is smaller for the in-house prepared column and this is consistent with the shorter retention times for the column. A smaller peak width provides a better chromatographic separation and is always a goal with liquid chromatography. The consistency for each injection, regarding shape, confirms the stability of the columns, also observed for the retention times. The intensities differ a bit more, but this could be due to the spray (stability, ion suppression) or the amount of ions the MS were able to trap within the 500 ms it was allowed to collect ions. This should be dealt with at a later stage when the method is used for quantitative purposes, e.g. with internal standards, but was not a concern at this stage. The commercial column shoved an increased signal intensity compared to the in-house column. Especially for low abundant proteins, as CYP27a1, the recovery (amount of analyte detected compared to analyte injected) is important. A drop in intensity could make the difference in detection or no detection when concentration is low. The peak area is a result of the peak width and peak intensity and is only shown to confirm this correlation. As the commercial column gave higher peak width and peak intensity it should provide higher peak area, as it does.

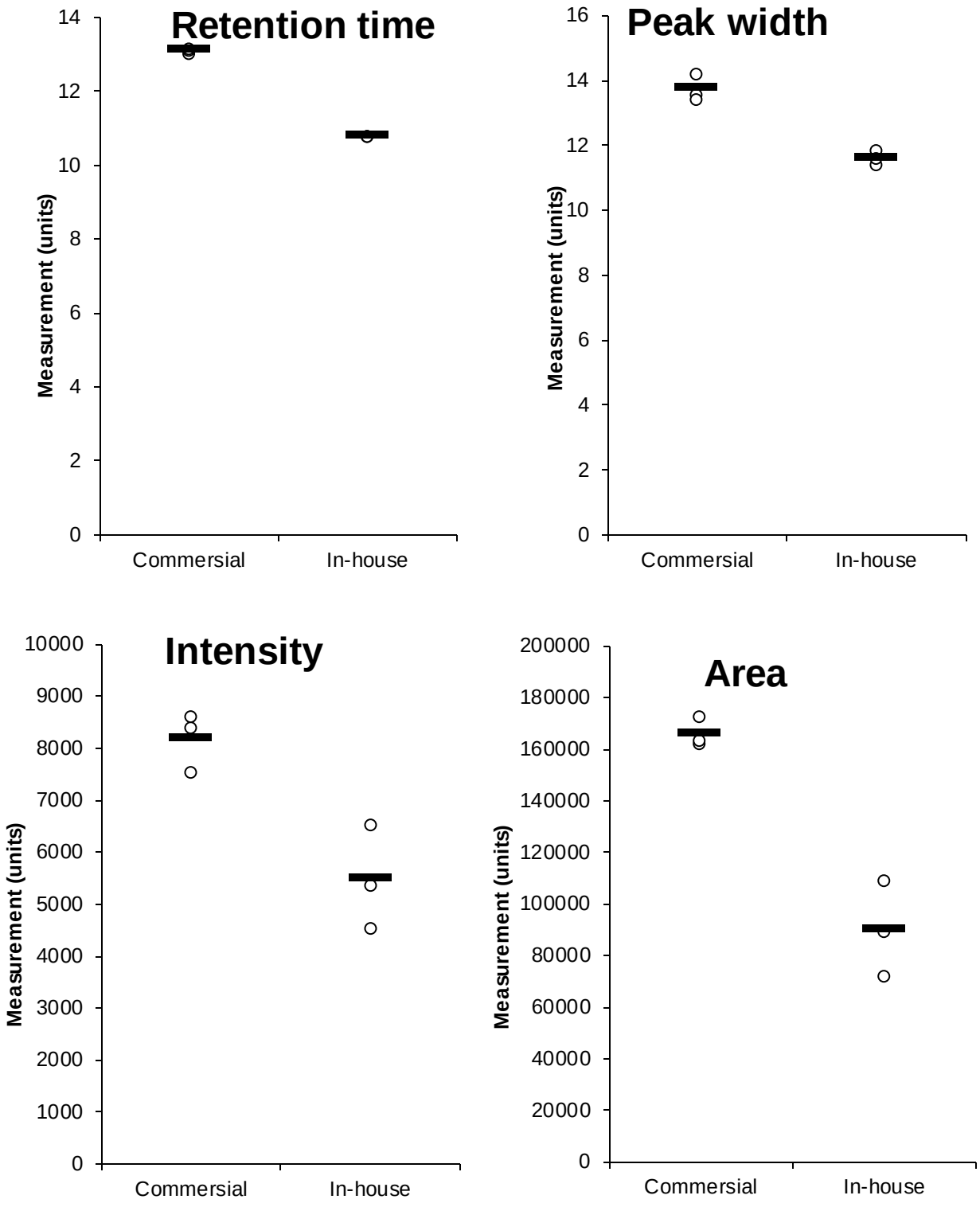


Figure 30 Plot of the comparison of a commercial precolumn with an in-house produced silica monolithic column. *The sample was injected three times for each column, indicated by the white dots. Each dot represents the average values for all six CYP27a1 fragments in the same replicate. The black line indicates the average value of the column.*

Based on the intensities, the commercial column should be the optimal choice as CYP27a1 is a low abundant protein. However, if an adequate sample preparation step could be established

for CYP27a1, e.g. the IP which is proven to work for beta-catenin, the in house column is a preferred choice. It provides both shorter retention times and peak width, and the cost-savings by using an in-house produced column is substantial compared to buying a commercial column. At this stage, choosing the in-house column will affect the detection of CYP27a1 due to lower intensities. However the in-house column was chosen for the further investigations due to better chromatography and lower cost.

Thus, the in-house produced silica-based C8 monolithic precolumn was used in further investigations.

6.6.3 Detection of CYP27a1 in cell samples

To evaluate the final method, two MDA-MB-231 cell samples with different sample preparation were investigated by targeted LC-MS; one CYP27a1 IP sample prepared by the established procedure for beta-catenin (***Figure 25***) , and one SDS-PAGE sample (no other sample treatment in prior).

MDA-MB-231 SDS-PAGE samples

For the gel-only sample, three gel lanes were combined before in-gel digestion to increase the CYP27a1 amount (figure). The LC-MS chromatograms with corresponding mass spectra are shown in ***Figure 31.***

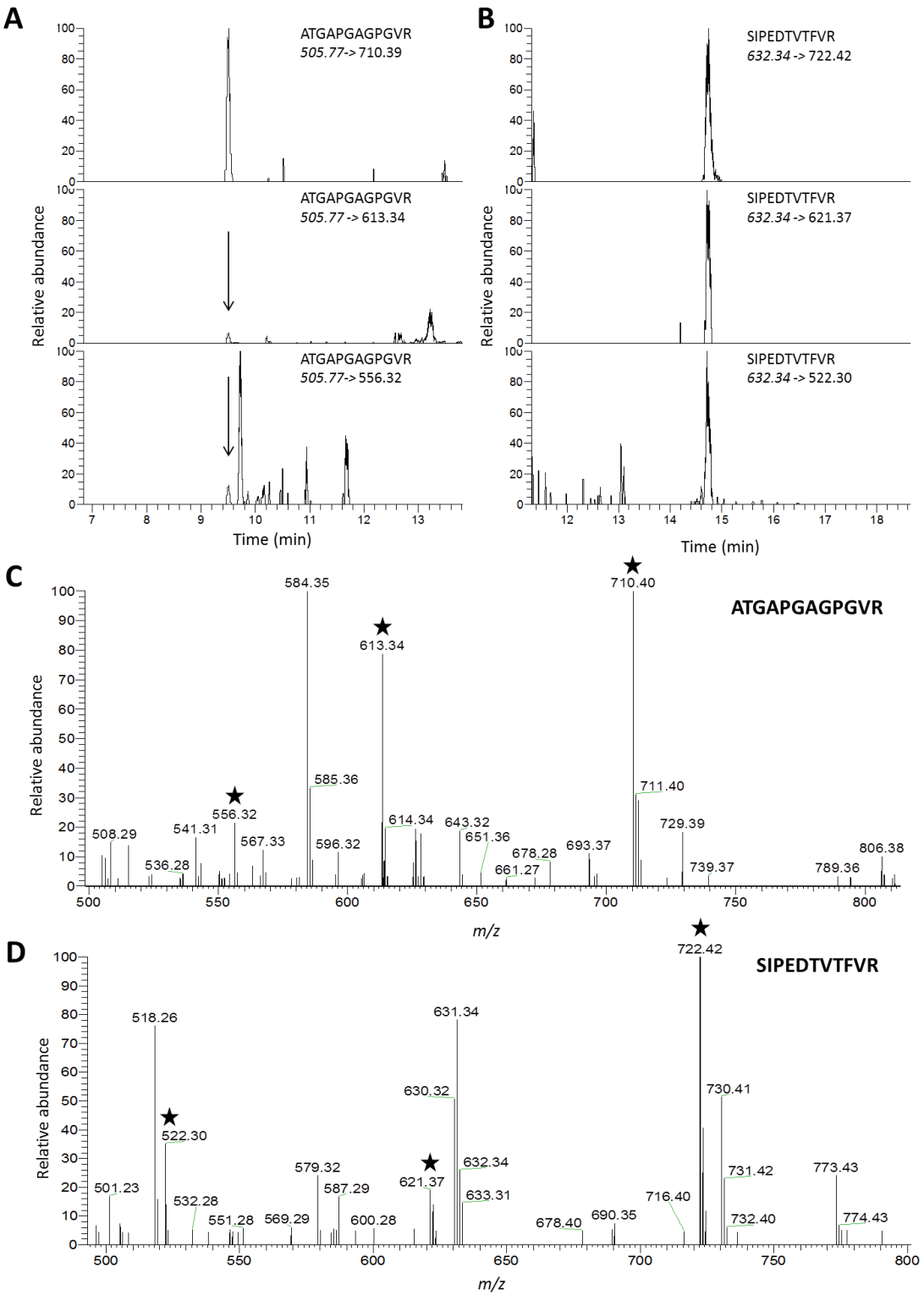


Figure 31A-B) LC-MS chromatograms of CYP27a1 in MDA-MB-231 gel samples. *The three fragments for the signature peptides A) ATGAPGAGPGVR and B) SIPEDTVTFVR are extracted in the chromatograms. In A, the peak corresponding to the fragment is marked with an arrow. C) The mass spectrum for ATGAPGAGPGVR. D) The mass spectrum for SIPEDTVTFVR. The corresponding m/z values for the fragments are marked with a star. The PRM method for CYP27a1 and the silica-based C8 monolithic precolumn were used. The analytical column and the LC conditions were the same as in* ***Figure 24.***

Two of the signature peptides SIPEDTVTFVR and ATGAPGAGPGVR were detected in MDA-MB-231 cell samples, by tMS/MS. The detections were based on the three fragments previously described (***Table 5***). The gel sample was injected six times to evaluate the repeatability, and the retention times were stable, with less than 2 % in relative standard deviation for both peptides. For each injection the peptides increased some in retention time, but this could be due to the analytical column, which had not been tested for many-injection stability [88]. For the ATGAPGAGPGVR target *m/z*, more intensive peaks occurred in the chromatograms, but they did not co-elute with the targeted peptide, and could hence be ignored. Sharp, intense peaks occurred for SIPEDTVTFVR, with less tailing than previously observed. For both peptides the fragment *m/z* were observed in the mass spectra (marked with a star in ***Figure 31C and D***).

The number of points per peak for ATGAPGAGPGV in the gel samples (***Figure 32A***) did not exceed the number of 8-10 points required for quantitative determination [99-101] (the number of points are discussed, and some also require up to 20-30 points per peak [102, 103]), but identification could be made due to robustness in retention time. The SIPEDTVTFVR peaks achieved adequate points per peak (***Figure 32B***). In the PRM method, the injection time (amount of time the MS collects ions before it scans) was set to 500 ms in order to obtain enough ions for detection. This, in addition to that MS scheduling was not set and the MS looked for all three signature peptide precursor ions throughout the entire gradient, loss of ions is a potential risk. To achieve more points per peak, a MS scheduling could be implemented in the PRM method, defining the retention time window for each peptide. This enables the MS to only look for one analyte ion at a time (if the analytes are separated) removing the risk of loss of ions because the MS is "busy" collecting or scanning another ion. The injection time could also be lowered, but for low-abundant analytes this could affect the detection (as fewer ions are collected before scanning). The last peptide (LLKPAEAALYTDAFNEVIDDFMTR) was not detected.

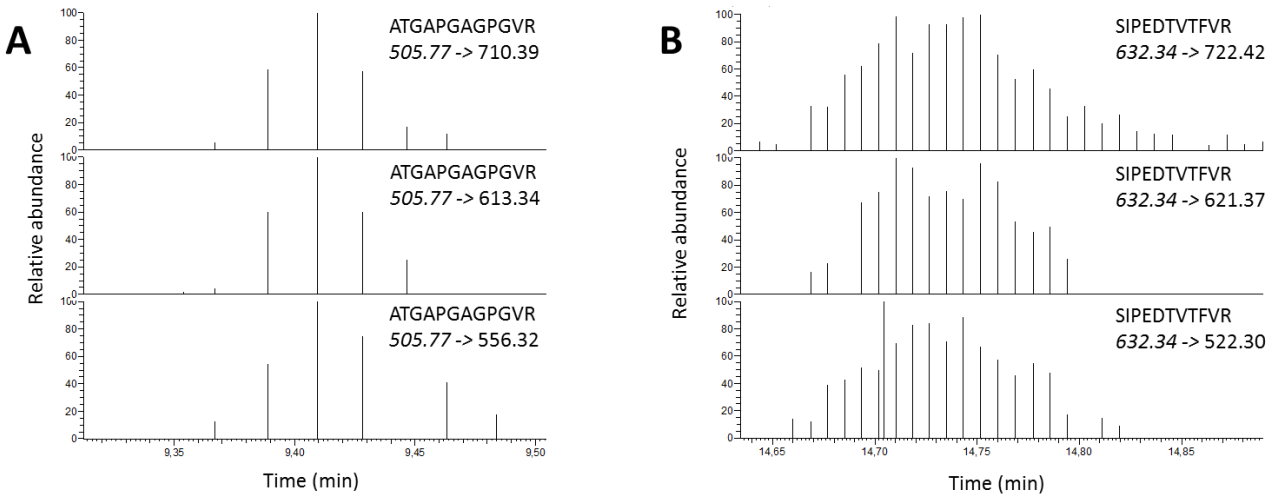


Figure 32 Chromatographic peaks presented as sticks (Thermo termination) for A) ATGAPGAGPGVR and B) SIPEDTVTFVR in a MDA-MB-231 gel sample. *The peaks are extracted without the use of peak smoothing, and illustrated as sticks in order to evaluate the number of points achieved for each fragment peak. The MS and chromatographic conditions used was the same as in Figure 31.*

Thus, CYP27a1 was identified in MDA-MB-231 samples, prepared by SDS-PAGE.

HEK293 IP samples

Even though the WB did not provide detection of CYP27a1 in IP samples, it was of interest to investigate the IP samples by the LC-MS method. The H 2 and H 2 ON samples from ***Figure 22*** were, after further preparations (***Figure 25***), analysed once each by LC-MS (tMS/MS-mode). A HEK293 IP sample was used instead of the MDA-MB-231 IP sample because the former IP investigations were for the mostly performed using HEK293 cells. In the WB performed for these samples (**Figure 22D**) the H 2 ON sample, which is the cell lysate from the overnight incubation in the IP procedure, gave a CYP27a1 band, while CYP27a1 was not detected in the H 2 sample (the citric acid extract in the IP procedure). When analysed by LC-MS however, the opposite results was found; CYP27a1 was detected in the H 2 sample but not in the H 2 ON sample. Chromatograms and mass spectra for SIPEDTVTFVR are shown in ***Figure 33.*** No peaks were achieved for ATGAPGAGPGVR, and the CYP27a1 detection was only based on one peptide. As the identification was the opposite of those achieved by WB, only one peptide was identified and only one injection was performed on the gel sample, no final conclusion could be made for the CYP27a1 IP method. These findings should be re-investigated.

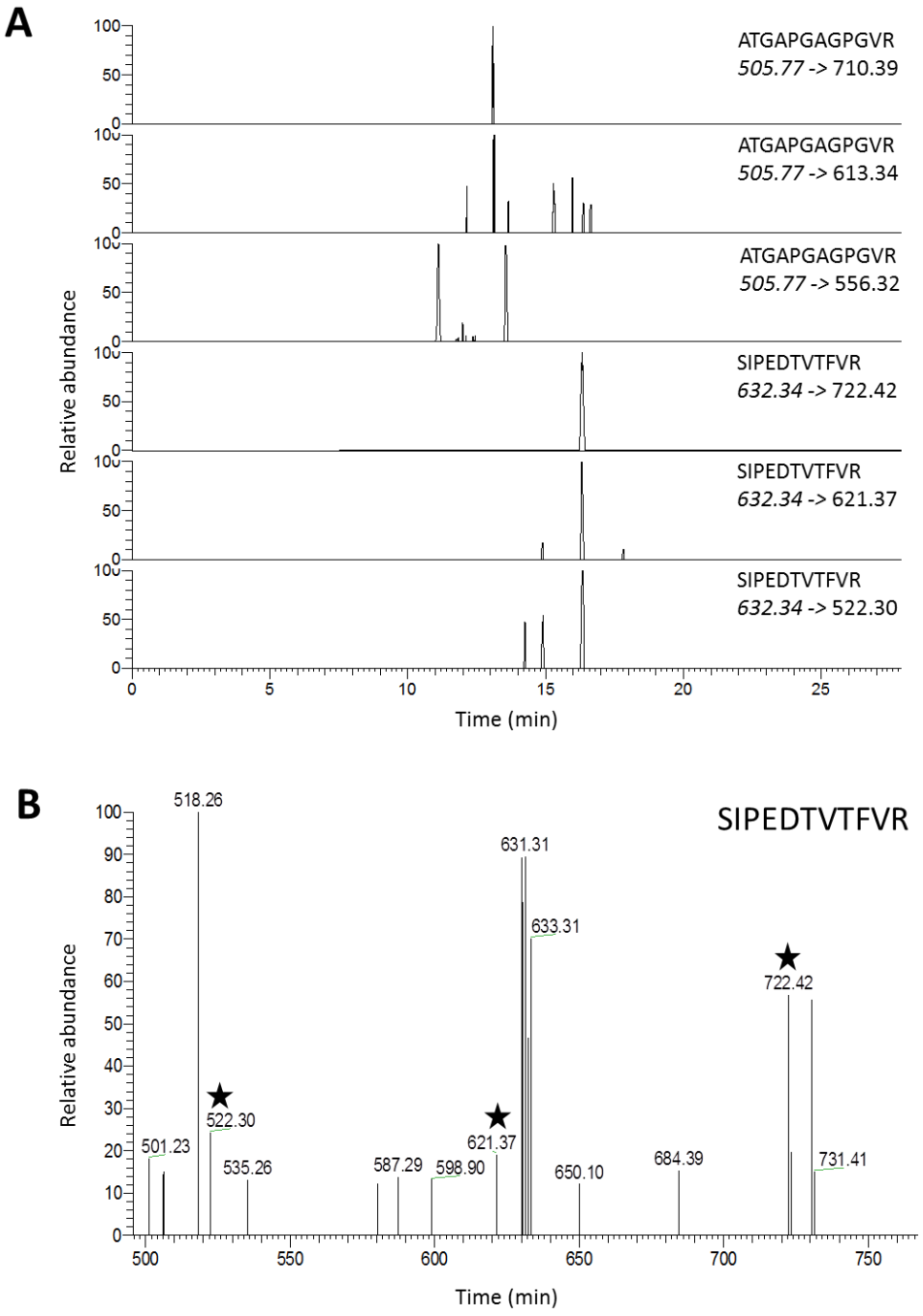


Figure 33 Chromatogram of CYP27a1 signature peptides (A) with mass spectra for SIPEDTVTFVR (B) in a HEK293 CYP27a1 IP sample. *The sample is prepared according to the established IP method for beta-catenin, including IP, SDS-PAGE, in-gel digestion and SPE. The PRM method for CYP27a1 (with retention time window implemented) and the silica-based C8 monolithic precolumn were used. The analytical column and the LC conditions were the same as in* ***Figure 24***

Thus, CYP27a1 was detected by LC-MS in IP-CYP27a1 antibody gel samples in preliminary investigations.

The choice of the signature peptides chosen in this thesis could be discussed, and implementing more signature peptides in the method could be performed, e.g. also using the signature peptides established by other researchers [72]. However, SIPEDTVTFVR and ATGAPGAGPGVR ensured a specific detection of CYP27a1, which were the goal of this thesis.

6.7 Can an analytical chemist help? My personal view on analytical biology.

A large part of this master thesis was performed using biological experiments in a biology lab. In order to be fully able to assess a workflow when analysing biological samples, an operator should be familiar with all aspects of the sample preparation. The production of cells, the sample clean-up procedures, and knowledge of which reagents are used throughout the process is of important to be able to fully investigate how to determine a biological analyte, e.g. a protein. Reagents applied in biological experiments e.g. detergents may mismatch with LC-MS instrumentation.

This experience, working with analytical biology and biological experiments has enabled me to see what biology can learn from an analytical chemist. A method development including validation, for LC-MS determination of a protein is time consuming, but ensures reproducibility. In biology they are dependant of reagents produced in living organisms which are prone to reproducibility issues, compared to that of synthetically produced chemicals in analytical chemistry. For example with an antibody, used for detection e.g. in WB, the performance may differ between the producers, and even between batches. Therefore, an antibody should be validated throurogly (e.g. by analysing protein knock-down samples compared to untreated samples) at every lab, and every time a new antibody is bought. This, however, is often not performed and the reproducibility is by that not secured. So even though the LC-MS validation is time consuming, the method developed could be much more robust between labs and chemical batches used.

In addition, the use of actin as a loading control is discussed [104-112]; calibration of the entire method is not provided, compared to that of analytical chemistry. It may be difficult to calibrate the method when working with e.g. cell culturing, but therefore the outputs (e.g. cell counting and WB protein comparisons) should be handled with more care. Loss of analyte during sample preparation is not measured accurately, and this also affect the robustness of

the determination, e.g. repeatability and reproducibility [42]. By implementing a "real" internal standard (not e.g. actin) and using that to determine the amount of analyte by calculations, the subjective "it seems to be a little less black" when evaluating WBs could be removed. A comparison of cell samples using LC-MS could be performed by metabolic labelling, e.g. stable isotope labeling with amino acids in cell culture (SILAC) [113-115].

7 Conclusion

The cytochrome P450 enzyme CYP27a1 was detected in MDA-MD-231 cell samples by a nano LC-MS/MS system using SDS-PAGE and subsequent in-gel digestion as sample preparation. The three peptides LLKPAEAALYTDAFNEVIDDFMTR, ATGAPGAGPGV and SIPEDTVTFVR were chosen as signature peptides and implemented in the PRM method for CYP27a1. Three fragment ions were identified for ATGAPGAGPGV and SIPEDTVTFVR, providing a specific detection of CYP27a1. The former peptide also provided adequate data for quantification. An in-house produced silica-based monolithic C8 column was assessed to be an optimal choice for the LC-MS analysis. Immunoprecipitation was investigated for enrichment of CYP27a1 prior to LC-MS analysis. This was not established for extraction of CYP27a1, but the method was successful targeting beta-catenin, hence proving the use of immunoprecipitation as an enrichment step prior to LC-MS analysis. Following establishment and validation of the LC-MS CYP27a1 method, e.g. sample stability, implementing an internal standard and robustness, this method could be a future tool for quantification of CYP27a1, enabling the study of CYP27a1 as a possible biomarker for ER+-breast cancer.

7.1 Future of the work

When a sample preparation method and a LC-MS method for quantification of CYP27a1 in cell samples is established and validated (e.g. internal standard is added and robustness is evaluated) the MCF7 cell line should be investigated as it is ER+, and knowledge of accurate levels of CYP27a1 are needed for an evaluation of the potential of CYP27a1 as an ER+-breast cancer biomarker. The CYP27a1 levels in macrophages and their correspondence to breast cancer should also be addressed.

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9 Appendix

9.1 Additional background

9.1.1 Cell culturing

How to grow cells

When working with cells, it must take place in a hood that provides sterile conditions. Cells are easily exposed to contaminations (e.g. bacteria) that affect cell behavior or also could cause cell death. A cell growth medium is used to provide the cells with necessary nutrition, and which medium used is dependent on cell type. Cells are kept in an incubator maintained at 37 ℃ and with 5 % CO_2, conditions that are supposed to replicate cells natural environment. When growing cells in a lab, different conditions could be altered to study a cells behavior, or to simply optimize cell growth; pH, concentrations of components such as glucose or phenol red, and the addition of growth factors or other additives. The growth factors stimulate the cellular growth, and fetal bovine serum (FBS) is the most used.

Cell culture passaging

In order to make working cell stocks, frozen cells that could be grown further, cells must be frozen in liquid nitrogen in growth media containing 5-10 % of dimethyl sulfoxide (DMSO). By adding DMSO, a slower cooling rate and a lower freezing point is obtained, reducing the risk of cell death due to ice crystal formation.

During cell growth, cells must be closely watched in order to contain optimal growing conditions. When cells are confluent (confluence describes the amount of growing area the cells cover), that they cover approximately 90 % of the growth area, they should be split (divided). When working with adherent cells (attached to a surface) this is performed by first loosen them from the surface they are attached to by adding a trypsin. When working with cells grown in-suspension the trypsin is not necessary as the cells floats in the medium. A small amount of the cells are then transferred to a new flask, and could continue to grow in freshly added growth media.

9.1.2 Transfection

Producing the DNA

As DNAs are large in size, an engineered plasmid, a circular double-stranded DNA, is used in transfection. These plasmids (vectors) are implemented into Escherichia coli (E. coli) bacterial cells and by growing these cells, the DNA to be used in transfection is produced. See Figure (AP) 1 for an overview. One part of the vector, the ORI, enables replication (they duplicate during cell dividing). Without this, cells implemented with a plasmid will not divide. The media/agar in which the bacterial cells are grown contains antibiotic, often ampicillin. To be able to grow in this media, the vector must contain a drug-resistant-part, meaning that this part makes the cell resistant to the antibiotic, and enables cell growth. A part of a DNA is then inserted into this vector, which is again inserted into an E.coli cell. During cell division the DNA, encoding for e.g. a target protein, is produced and can afterwards be extracted from the cells and transferred into mammalian cells.

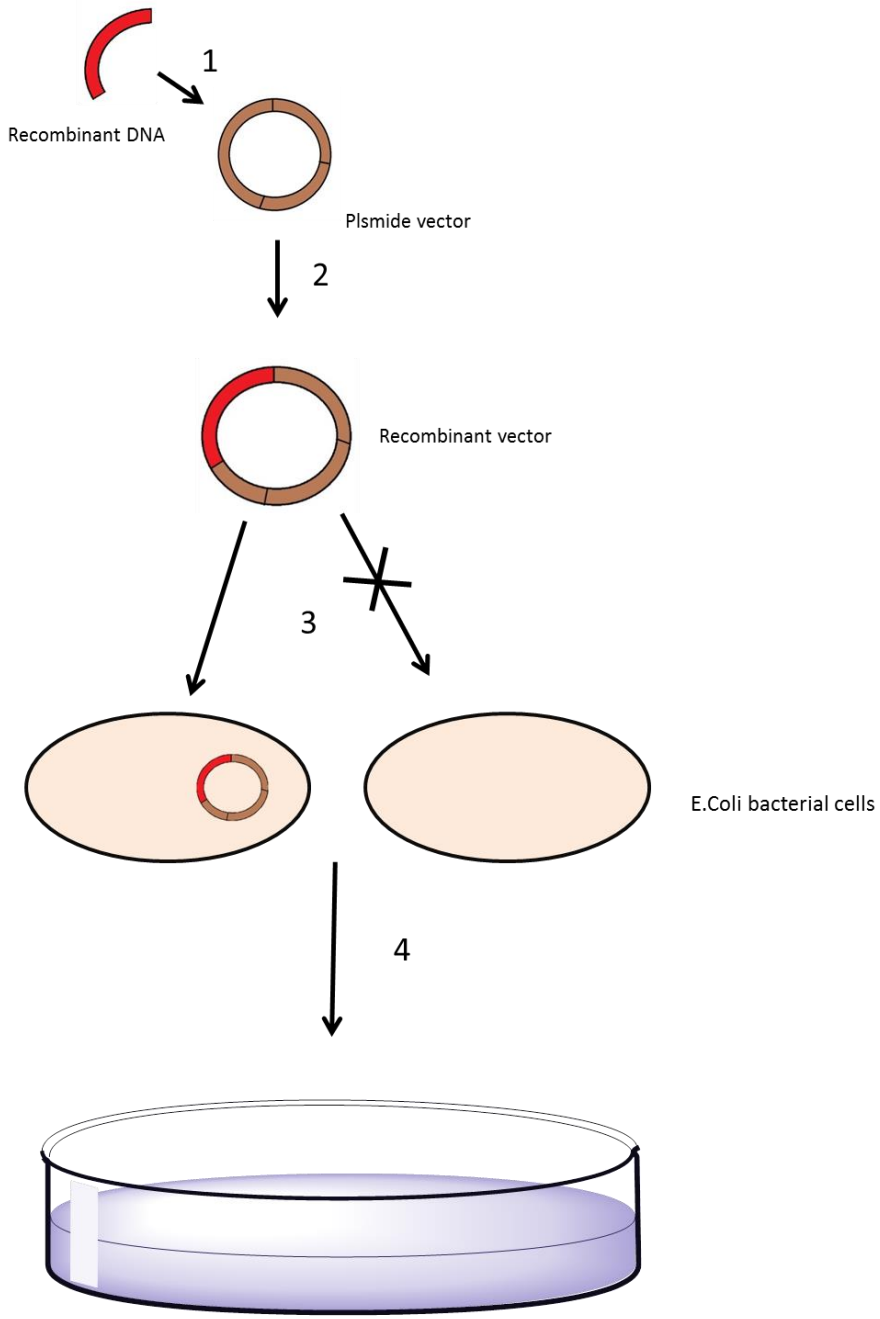

Figure (AP) 1 A scheme of the procedure for transformation. *A recombinant DNA is incorporated into a plasmid vector, also containing an ORI part enabling growth, and an antibiotic resistant part. By implementing into an E.coli cell, the DNA is produced, and could later be extracted from the cell and used in transfection.*

Inserting DNA into cells

Different methods are used to transport the DNA into the cell; chemical, physical and biological. The biological method is virus mediated, while the physical method uses physical tools as laser or direct micro injection. Chemical transfection is most used, and is the method used in this thesis. Here, the DNA binds to a chemical reagent that carries it across the cell

membrane (exact mechanism is not known). An example of a chemical reagent is calcium phosphate.

9.2 Tables

Table (AP) 1 Components, amounts and final concentrations of NP-40 lysis buffer

1 M Tris pH 8	*2.5 mL*	*50 mM*
5 M NaCl	*1.5 mL*	*150 mM*
NP-40	*0.5 mL*	*1 %*
EDTA	*200 µL*	*2 mL*
MQ-water	*43.4 mL*	

Table (AP) 2 Components, amounts and final concentrations of 5x loading buffer.

1 M tris-HCl pH 6.6	0.6 mL	60 mM
50 % Glycerol	5 mL	25 %
10 % SDS	2 mL	2 %
2-Mercaptoethanol	0.5 mL	14.4 mM
1 % Bromophenol blue	1 mL	0.1 %
Water	0.9 mL	

Table (AP) 3 Components and amounts of 10 x transfer buffer.

Trizma – base	30.3 g
Glysin	144.0 g
Water	Dilute to 1000 mL

Table (AP) 4 Protein sequences of CYP27a1 and beta-catenin.

Protein	Sequence
Beta-catenin	MSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDKWRNKKFELGLEFPNLPYYI DGDVKLTQSMAIIRYIADKHNMLGGCPKERAEISMLEGAVLDIRYGVSRIAYSKDFETLK VDFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDALDVVLYMDPMCLDAFPKL VCFKKRIEAIPQIDKYLKSSKYIAWPLQGWQATFGGGDHPPKSDLEVLFQGPLGSATQAD LMELDMAMEPDRKAAVSHWQQQSYLDSGIHSGATTTAPSLSGKGNPEEEDVDTSQVLY EWEQGFSQSFTQEQVADIDGQYAMTRAQRVRAAMFPETLDEGMQIPSTQFDAAHPTNV QRLAEPSQMLKHAVVNLINYQDDAELATRAIPELTKLLNDEDQVVVNKAAVMVHQLSK KEASRHAIMRSPQMVSAIVRTMQNTNDVETARCTAGTLHNLSHHREGLLAIFKSGGIPA LVKMLGSPVDSVLFYAITTLHNLLLHQEGAKMAVRLAGGLQKMVALLNKTNVKFLAIT TDCLQILAYGNQESKLIILASGGPQALVNIMRTYTYEKLLWTTSRVLKVLSVCSSNKPAI VEAGGMQALGLHLTDPSQRLVQNCLWTLRNLSDAATKQEGMEGLLGTLVQLLGSDDIN VVTCAAGILSNLTCNNYKNKMMVCQVGGIEALVRTVLRAGDREDITEPAICALRHLTSR HQEAEMAQNAVRLHYGLPVVVKLLHPPSHWPLIKATVGLIRNLALCPANHAPLREQGAI PRLVQLLVRAHQDTQRRTSMGGTQQQFVEGVRMEEIVEGCTGALHILARDVHNRIVIRG LNTIPLFVQLLYSPIENIQRVAAGVLCELAQDKEAAEAIEAEGATAPLTELLHSRNEGVAT YAAAVLFRMSEDKPQDYKKRLSVELTSSLFRTEPMAWNETADLGLDIGAQGEPLGYRQ DDPSYRSFHSGGYGQDALGMDPMMEHEMGGHHPGADYPVDGLPDLGHAQDLMDGLP PGDSNQLAWFDTDL
CYP27a1	KATGAPGAGPGVRRRQRSLEEIPRLGQLRFFFQLFVQGYALQLHQLQVLYKAKYGPMW MSYLGPQMHVNLASAPLLEQVMRQEGKYPVRNDMELWKEHRDQHDLTYGPFTTEGH HWYQLRQALNQRLLKPAEAALYTDAFNEVIDDFMTRLDQLRAESASGNQVSDMAQLF YYFALEAICYILFEKRIGCLQRSIPEDTVTFVRSIGLMFQNSLYATFLPKWTRPVLPFWKR YLDGWNAIFSFGKKLIDEKLEDMEAQLQAAGPDGIQVSGYLHFLLASGQLSPREAMGSL PELLMAGVDTTSNTLTWALYHLSKDPEIQEALHEEVVGVVPAGQVPQHKDFAHMPLLK AVLKETLRLYPVVPTNSRIIEKEIEVDGFLFPKNTQFVFCHYVVSRDPTAFSEPESFQPHR WLRNSQPATPRIQHPFGSVPFGYGVRACLGRRIAELEMQLLLARLIQKYKVVLAPETGEL KSVARIVLVPNKKVGLQFLQRQC

Table (AP) 5 Amino acid abbreviations

Abbreviation		Amino acid name
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

Table (AP) 6 Signature peptides with m/z of corresponding precursor ions and fragments, for beta-catenin. From [96].

Beta-catenin peptides	Precursor $[M+2H]^{2+}$ *m/z*	Fragment *m/z*				
LLNDEDQVVVNK	693.36731	y_4^+ 459.2929	y_7^+ 801.447	y_8^+ 930.4899	y_9^+ 1045.5161	y_{10}^+ 1159.5590
HAVVNLINYQDDAELATR	681.35327	y_8^+ 890.4222	y_9^+ 1018.481	y_{10}^+ 1181.544	y_{11}^+ 1295.588	
NEGVATYAAAVLFR	741.39801	y_7^+ 747.4519	y_8^+ 910.5156	y_{19}^+ 1011.563	y_{10}^+ 1082.6	
ATVGLIR	365.2345	y_4^+ 458.309	y_5^+ 557.3776			

Table (AP) 7 Signature peptide investigations. *CYP27a1 peptides listed as highly observable in PeptideAtlas was investigated further. In the first lane, all peptides not containing C, W or M are marked with dark grey. In the unique-column dark grey indicates 100 % uniqueness, while light grey indicates that the peptide matches to a certain percentage to other proteins (but not 100 % match). White means not unique. The three lanes 1, 2 and 3 represent three CYP27a1 standard LC-MS analysis. White colour means that the peptide was not found in proteome discoverer. Light grey indicates that the found is inadequate (e.g. only one detected fragment ion), and dark grey indicates adequate discovery with enough detected fragment ions for identification.*

Peptide	Modification residues	Unique	1	2	3
AAIPAALPSDK					
ATGAPGAGPGVR					
DFAHMPLLK					
EIEVDGFLFPK					
IAELEMQLLLAR					
IVLVPNKK					
LLKPAEAALYTDAFNEVIDDFMTR					
NDMELWK					
NSQPATPR					
SIGLMFQNSLYATFLPK					
SIPEDTVTFVR					
VVLAPETGELK					
WTRPVLPFWK					
YLDGWNAIFSFGK					
DPEIQEALHEEVVGVVPAGQVPQHK					

IQHPFGSVPFGYGVR					
DPTAFSEPESFQPHR					
LEDMEAQLQAAGPDGIQVSGYLHFLLASGQLS PR					
DQHDLTYGPFTTEGHHWYQLR					
LYPVVPTNSR					
EAMGSLPELLMAGVDTTSNTLTWALYHLSK					
DFAHMPLLK					
VGLQFLQR					
NTQFVFCHYVVSR					
YGPMWMSYLGPQMHVNLASAPLLEQVMR					
FFFQLFVQGYALQLHQLQVLYK					
IVLVPNK					
SLEEIPR					
MAALGCAR					
IGCLQR					
AESASGNQVSDMAQLFYYFALEAICYILFEK					
QALNQR					
GLCPHGAR					

Table (AP) 8 Uniprot search for peptide uniqueness for SIPEDTVTFVR and ATGAPGAGPGVR. *Peptide sequence matches, with percentage match are found by blasting in Uniprot. Average masses are found in* http://www.peptidesynthetics.co.uk/tools/.

Peptide	Uniprot of matched protein	% match	SIPEDTVTFVR sequence	Average mass	Sequence of matched protein	Average mass
SIPEDTVTFVR	B4E2I9_HUMAN	66.7	PEDTVTFVR	1063.157	PDDVVTFIR	1061.184
	SEM3C_HUMAN	66.7	PEDTVTFVR	1063.157	PDDVVTFIR	1061.184
ATGAPGAGPGVR	D6RAC1_HUMAN	73.3	AT-GAPG--GPGVR	953.048	ATRGSPGRGAGPGVR	1395.526
	G3CIG0_HUMAN	88.9	ATGAPGAGP	697.729	ATGTPGAGP	727.755
	GXLT2_HUMAN	81.8	GA-PGAGPGVR	837.916	GALPGASPGVR	981.102
	F8WAH6_HUMAN	88.9	GAPGAGPGV	681.729	GAPGAPGV	624.677

9.3 Western blot raw files

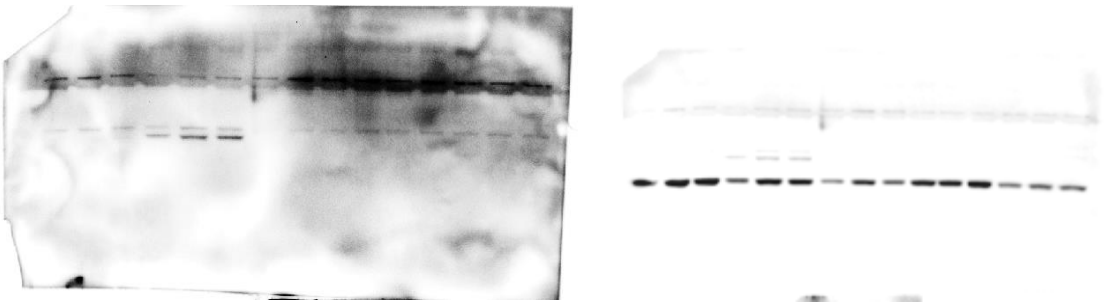

Figure (AP) 2 WB raw files for the CYP27a1 antibody incubation (left) and actin antibody incubation (right) in Figure 16A.

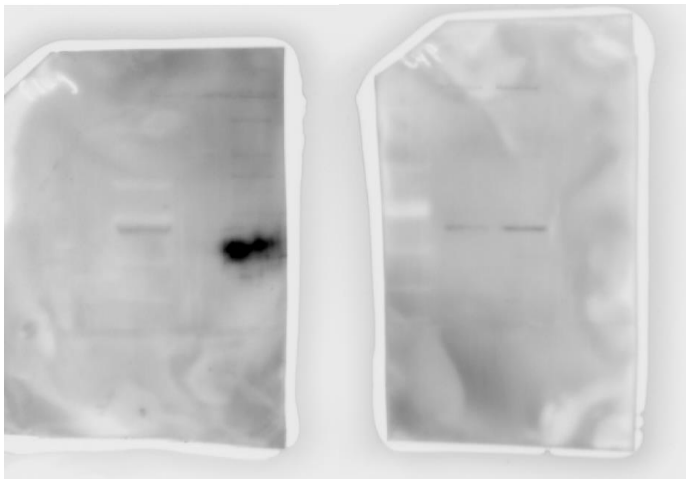

Figure (AP) 3 WB raw files for the FLAG antibody incubation (left) and CYP27a1-antibody incubation (right) in Figure 18.

Figure (AP) 4 WB raw file for Figure 20.

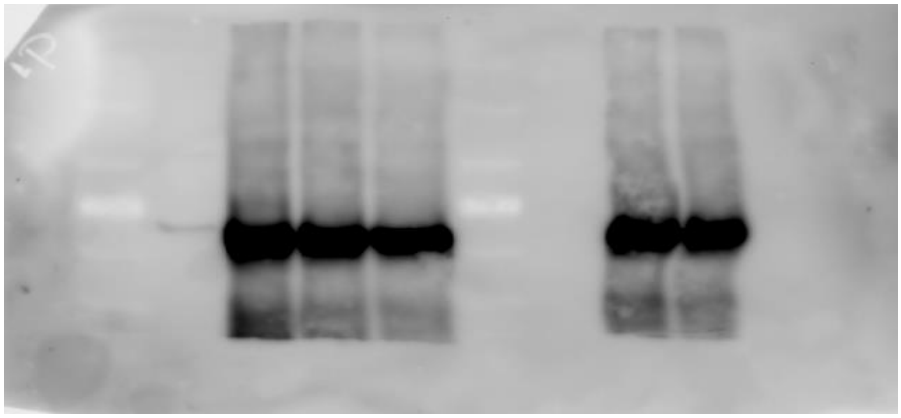

Figure (AP) 5 WB raw file for Figure 21

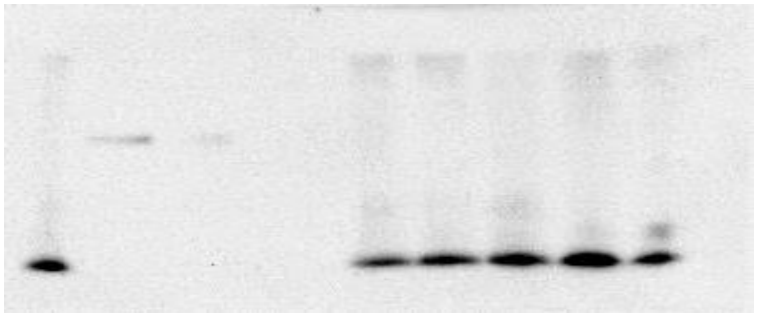

Figure (AP) 6 WB raw file of Figure 22B. *The two lanes to the right is the two first lanes in* ***Figure 22C*** *(M 1 and M 2).*

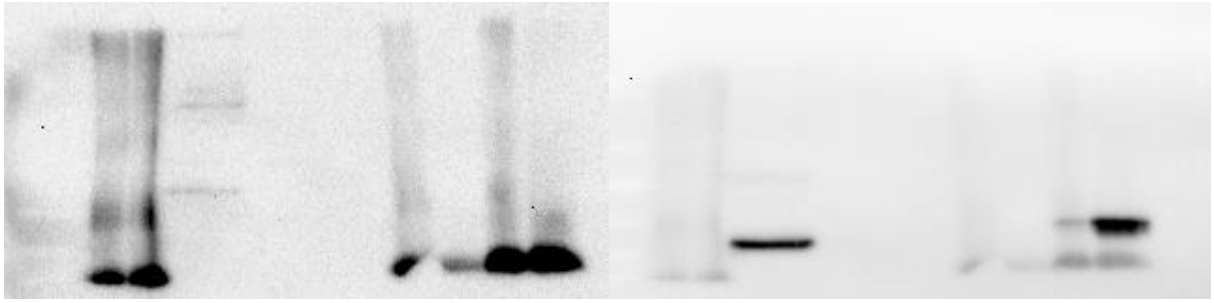

Figure (AP) 7 WB raw files (CYP27a1 to the left, and actin to the right) of Figure 22D. *The two lanes two the right is the two last lanes in* ***Figure 22C*** *(M 3 and negative control)*

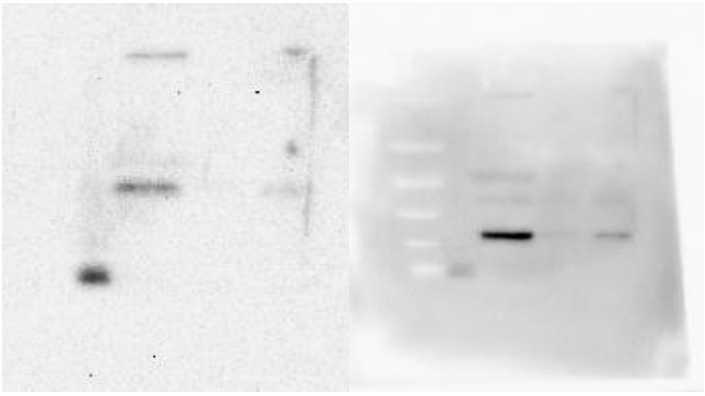

Figure (AP) 8 WB raw files for Figure 22E. *CYP27a1 to the left and actin to the right.*

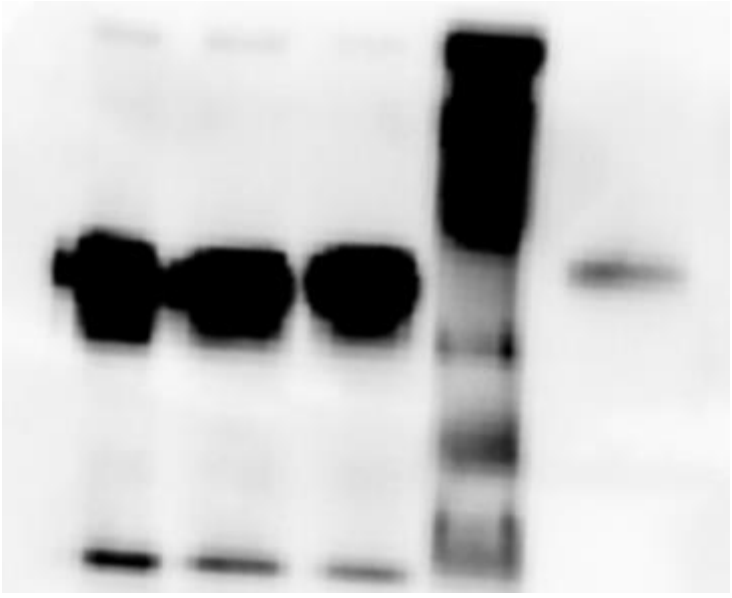

Figure (AP) 9 WB raw file for Figure 23.