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Open Tubular Enzyme Reactors (OTERs) for On-line Protein Digestion in Liquid Chromatography Mass Spectrometry Proteomics

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1 Preface

The presented work has been carried out at the Department of Chemistry at the University of Oslo (UiO) in the period from August 2012 to May 2014. My supervisors have been Ph.D. student Hanne Kolsrud Hustoft, Ass. Prof. Steven Ray Wilson, Prof. Tyge Greibrokk and Prof. Elsa Lundanes.

First, I would like to thank all my supervisors, especially Hanne K. Hustoft who introduced me to OTERs in Desember 2012, and has ever since been helpful in guiding me through this study. I am deeply humbled to be a part of this research project, which I hope will be further developed.

The OTERs developed in this study were integrated into a manual liquid chromatography (LC) mass spectrometry (MS) system developed by Hanne K. Hustoft, and this system was automated by Ph.D. student Tore Vehus. Testing of the OTERs was performed under the supervision of Hanne.

I would like to thank Caroline for good and kind conversations, for being a good friend throughout the bachelor and master program, and proofreading this thesis. Furthermore I would like to thank Dr. Magnus Røgeberg and Dr. Helle Malerød for helpful, absurd and interessting discussions during the first year of this master project. A special thanks to "Tore på kontoret" for developing the automated LC-MS-system and for good laughs in the office.

Inge Mikalsen have been a great help with the pressure bombs, and Marita Clausen by lending us lab equipment. Thank you Rena, Dorna and Silvija for good column production tips, and Hanne R.L., Dr. Sofia and Elin for helpful guidelines concerning general chromatography knowledge. Trude and Marianne have been great study companions throughout this master project. Per Sira and Magnus Taraldsen at USIT (UiO) have been helpful with professional filming and editing of the OTER production video.

Finally, I would like to thank my family and close ones for their support.

Oslo, Norway, May 2014

Ole K. Brandtzæg

2 Abbreviations and Definitions

ABC – ammonium bicarbonate

ACN – acetonitrile

AIBN - 2,2´azobis(2-methylpropinonitrile)

Arg – arginine

Asp – aspartic acid

AU – arbitrary units; a relative unit of measurement to show the ratio of quantity of specie.

Autodigestion – self-digestion or autolysis

BuMa - butyl methacrylate

CaA – carbonic anhydrase 2

Carry-over – traces of the previous sample in the system after an injection

CEC – capillary electrochromatography

C_{MAX} – maximum analyte concentration

Comprehensive proteomics – record MS/MS of all detected peptides

Cyt C – Cytochrome C

D – dilution

Dig. time – digestion time

D_m – diffusion coefficient

DMF – N,N-dimethylformamide

d_p – particle diameter

DPPH – 2,2-diphenyl-1-picrylhydrazyl hydrate

DTT – 1,4-dithiothreitol

DVB – divinylbenzene

E+S – enzyme to substrate ratio

EDMA – ethylene dimethacrylate

EIC – extracted ion chromatogram

Enzyme – reaction catalyzing protein

ES – electrospray

ES-MS – electrospray mass spectrometry

EtOH – ethanol

FA – formic acid

Fibr – fibrinogen

GC – gas chromatography

H – plate height

HCD – higher-energy collision dissociation

HEMA – 2-hydroxyethyl methacrylate

Hemo – Hemoglobin

HPLC – high performance liquid chromatography

HSA – human serum albumin

IAA – iodoacetic acid

IAM – iodoacetamide

ID – inner diameter

IgG – immunoglobulin G

IMER – immobilized enzyme reactor

k – retention factor

kDa – kilo Dalton, $gmol^{-1}$

L – column length

LC – liquid chromatography

LFD – large field detector

LOD- limit of detection

Lys – lysine

Lys-C – Endoproteinase Lys-C

m – mass

Mm – molar mass

MS – mass spectrometry

MS/MS – tandem MS

Myo – myoglobin

n – replicate injections

N – plate number

NH_4OAc – ammonium acetate

Nu – nucleophile

OD – outer diameter

On-line – Performing analysis “on-line” means that it is performed in the chromatographic system during the course of a sample workflow.

OT – open tubular

OTER – open tubular enzyme reactor

PGC – porous graphitized carbon

PNGase F – Peptide-N-Glycosidase F

Pore size – the diameter of the voids between the polymers of a monolithic structure

Pro – proline

ProGRP – progastrin-releasing peptide isoform 1

PS-DVB – polystyrene divinylbenzene

Rep – replicate

RSD % – relative standard deviation in percent

SEM – scanning electron microscope

SPE – solid phase extraction, also known as pre-column

SQ % - amino acid sequence coverage in percent of a protein that is identified.

St. Dev. – standard deviation

T/L – trypsin/endoproteinase Lys-C

tABC – triethyl ammonium bicarbonate

Targeted proteomics – rely on the selectivity of multiple reaction monitoring (MRM) for recognizing specific analytes.

Temp. - temperature

TFA – trifluoroacetic acid

TIC – total ion chromatogram

Transf – transferrin

u – mobile phase linear velocity

UHPLC – ultra-high performance liquid chromatography

UV – ultraviolet

VDM – vinyl azlactone

V_M – void volume

$W_{0.1}$ – peak width at 10 % of peak maximum

β-cat – β-catenin

γ-MAPS – 3-(trimethoxysilyl)propyl methacrylate

ΔP – pressure drop

η – viscosity

ϕ – flow rate resistance factor

μ_{OPT} – optimal linear velocity

3 Abstract

Sample preparation in “bottom-up” proteomics consists of denaturation, reduction and alkylation of the proteins, before enzymatic digestion of the proteins into peptides. The rate limiting step is the enzymatic digestion, and digestion overnight is recommended. Hence, for faster analysis, the digestion time should be reduced.

The main focus of this master thesis was the development of 20 μm inner diameter (ID) open tubular enzyme reactors (OTERs) based on 2-hydroxyethyl methacrylate-co-vinyl azlactone (HEMA-VDM) for “on-line” protein digestion of limited sample sizes in a nano liquid chromatography (LC) - mass spectrometry (MS) system. Monolithic solid phase extraction (SPE) pre-columns based on butyl methacrylate (BuMa) and polystyrene divinylbenzene (PS-DVB) were prepared in 50 μm ID capillaries and used for trapping of peptides generated by the OTER. These peptides were then separated using 10 μm ID PS-DVB porous layer open tubular (PLOT) columns.

The developed OTER was prepared by polymerization using a polymerization mixture consisting of HEMA and VDM as monomer, 1-heptanol or 1-decanol as porogen, and 2,2´azobis(2-methylpropinonitrile) (AIBN) as initiator. Short OTERs were prepared with 1-decanol as porogen, while for longer OTERs, 1-heptanol was used.

The sample was loaded onto the OTER with a loading buffer consisting of 50 mM NH_4OAc pH 8.75 with 4 % acetonitrile (ACN). A trapping time of 4 min from the OTER to the SPE column was found to be optimal (for the short OTER) using the manual LC-MS-system (developed by Hanne K. Hustoft). The run-to-run retention time repeatability in this system, was 0.25-0.44 % in relative standard deviation (RSD %), and 300 attomoles of targeted recombinant progastrin-releasing peptide isoform 1 (ProGRP) could be detected. The developed long OTER, immobilized with Trypsin/endoproteinase Lys-C (T/L) gave sequence coverages (SQ %) up to 95 % of standard proteins. The optimal reactor temperature during digestion and the optimal digestion time was 37 $^{\circ}$C and 30 min, correspondingly. The OTER could be integrated in an automated LC-MS-system (Hanne K. Hustoft and set up by Tore Vehus), where the within and between digestion repeatability were satisfactory. About 1500 proteins were identified in a single analysis when injecting 1 μg of a human cell lysate sample using the OTER in the automated system.

4 Introduction

4.1 Proteomics

The word “proteome” was introduced by Wilkins et al. [1] in 1996, describing the content in a living cell or an organism related to the proteins expressed by the genome. In 1998, Anderson et al. [2] defined proteomics as “the use of quantitative protein-level measurements of gene expression to characterize biological processes (e.g., disease processes and drug effects) and decipher the mechanism of gene expression control”. The variation in the abundance and properties of proteins will be an aid in the quest of observing the functionalities they possess. Compared to the genome, the proteome is more variable, and the protein expression varies between cell type, tissue, physiological and environmental conditions. The complexity of proteins increases after translation. This complexity is caused by processes such as post translational modifications, alternative splicing, cleavage, and break-down products [3]. The dynamic range can exceed 10^{10} in plasma [4] after undergoing these processes.

The most used detection technique in proteomics is MS, but the challenge when using this method is that the dynamic range is only 10^4 in a single spectrum [5,6], and overlap of proteins, or peptides, occur. Therefore, proteins, or peptides, need to be separated before identification and quantification because this results in increased dynamic range. The two ways of studying proteomics are “top-down” and “bottom-up” (Figure 1).

The top-down approach consists of separation and detection of intact proteins. The samples in this approach cannot be very complex, and the disadvantages with top-down are limited resolution and recovery, carry-over issues, and the need of a high-end MS for protein identification [7-9]. However, top-down can give SQ % up to almost 100 %, and gives more information about post translational modifications compared to bottom-up [10]. High sequence coverage can also be obtained by the bottom-up approach, which is most used today, where proteins are digested to peptides that are subsequently separated and detected by LC-MS.

Bottom-up can reveal important information regarding post translational modifications or sequence variants, while in top-down the intact protein masses and fragment ion masses, due

to gas-phase dissociation, are measured. However, at present the bottom-up approach is preferred because of more efficient chromatography and MS detection [11].

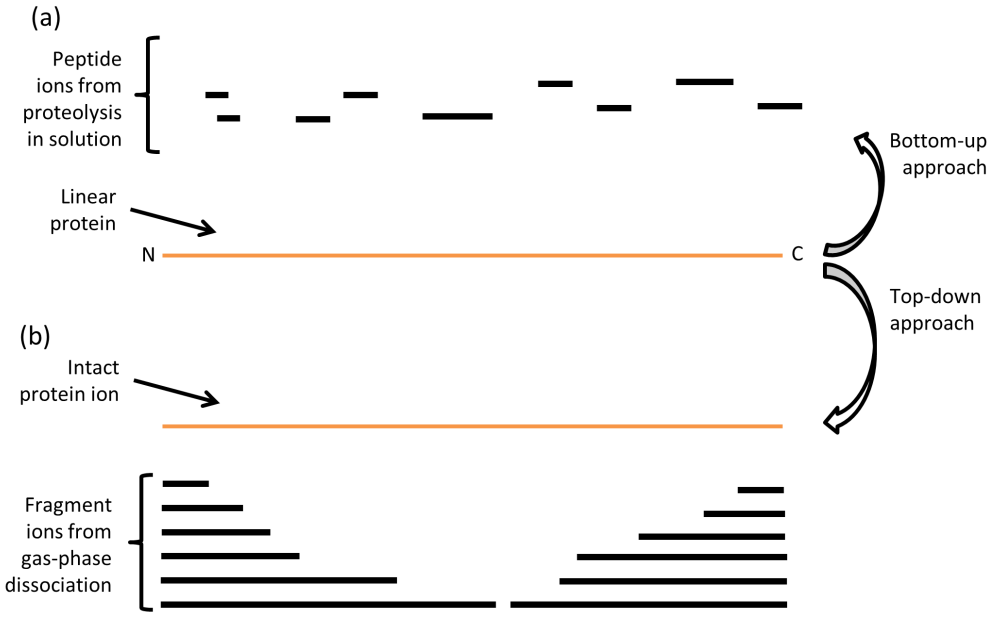

Figure 1: (a) Bottom-up and (b) top-down approaches for protein sequence analysis. The identified protein sequence in top-down and bottom-up can reach 100 %. Figure adapted from [10].

Bottom-up proteomic based experiments consists of five stages (Figure 2). In stage 1, proteins are dissolved in an appropriate solvent. The proteins are degraded enzymatically (or chemically) to peptides in stage 2, and the enzyme utilized in this step is usually trypsin. Others have reported using endoproteinase Lys-C (Lys-C) [12], and a combination of Lys-C and trypsin [13] in order to achieve smaller peptides and fewer missed cleavages. Enzymatical degradation with trypsin (see section 4.5) results in peptides with C-terminally protonated amino acids. This is an advantage in peptide sequencing. The peptides can be separated by LC and eluted into an electrospray (ES) ion source in step 3. Very fine droplets are produced in the ES ion source and these are evaporated between the ES and MS. Multiple protonated peptides enter the MS. In stage 4, a MS spectrum of the eluting peptide is recorded one by one. In step 5, the MS software produces a prioritized list of these peptides for fragmentation before a sequence of tandem MS (MS/MS) experiments proceeds. These experiments include isolation and fragmentation by collision with energetic gas for a given peptide ion, followed by recording of the MS/MS spectrum. MS and MS/MS spectra are stored so that they can be matched against those from protein sequence databases. The resulting outcome of the

experiment is to identify the peptides, and to match these peptides to their corresponding proteins [14].

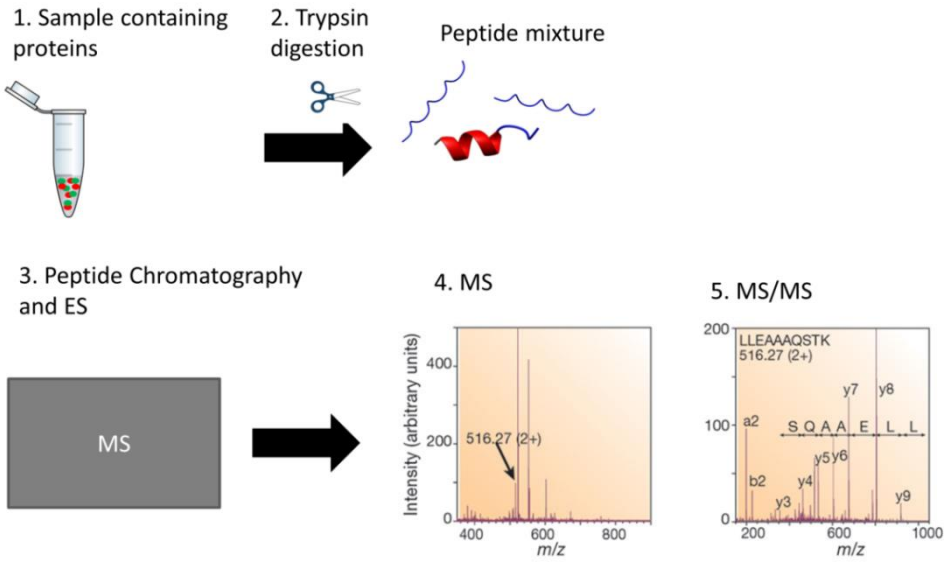

Figure 2: The 5 steps of bottom-up proteomics. Adapted from Aebersold et al. [14].

4.2 High performance liquid chromatography

As stated above, LC is used to separate the peptides, which are generated from digested protein samples, in columns before MS detection. In modern time, the importance of being able to analyze species in complex samples is tremendous.

In 1941, Martin and Synge [15] became aware of that particle packed separation columns require very small particles to provide high chromatographic efficiency, and that a high pressure is crucial for driving the mobile phase through the column. Hence, LC became high pressure liquid chromatography, also known as high performance liquid chromatography (HPLC). The stationary phases in HPLC columns can be of various formats (see section 4.4) for different analytes.

As the need for analyzing smaller samples emerged, narrow columns, and pumps that are able to deliver low flow rate became necessary. Microcolumn LC was introduced by Horváth and co-workers in 1967 [16] when they separated ribonucleotides on a pellicular particle packed stainless steel column (0.5-1.0 mm ID). This was a breakthrough in microcolumn LC as the

sample volume and the volumetric flow rates were reduced. Hence, less dilution (D) of the sample in the column results in improved detection limits with concentration sensitive detectors, such as is electrospray mass spectrometry (ES-MS) [17]. In addition to that, less consumption of mobile phase is an advantage for our environment. The ID of different classification of columns is listed in Table 1.

Table 1: ID of the different classification of columns. Adapted from [18]

Column designation	Typical ID [mm]
Conventional HPLC	3-5
Narrow-bore HPLC	2
Micro LC	0.5-1
Capillary LC	0.1-0.5
Nano LC	0.01-0.1
OT LC	0.005-0.05

However, microcolumn LC puts larger demands to the operator; to avoid dead volumes in miniaturized LC. In addition to that, miniaturized LC is not as robust as conventional LC as the possibility of clogging is an increasing factor.

Changing the particles size from 3.5-5 μm in HPLC to < 2 μm in ultra-high performance liquid chromatography (UHPLC), enhance sensitivity, speed and efficiency with respect to HPLC [19]. The easiest way to improve the efficiency (plate number, N) of a column is to reduce the plate height (H) and increase the column length (L). For particle packed columns, H can be reduced by reducing the particle diameter (d_p).

$$N = \frac{L}{H} \approx \frac{L}{2d_p} \quad \text{Eq. 1}$$

Reducing H and increasing L is limited by the pressure drop (ΔP) over the column, which is given by:

$$\Delta P = \frac{uL\eta\phi}{d_p^2} \quad \text{Eq. 2}$$

where ϕ is the flow rate resistance factor, u is the mobile phase linear velocity, η is the mobile phase viscosity, and L is the length of the column. ΔP is inversely proportional to the square of the particle diameter. When the flow rate is kept constant, a decrease in particle size, or increase in column length by a factor of two, give an increase in pressure by four and two,

respectively. Furthermore, the optimal linear velocity (μ_{opt}) is inversely proportional to the particle diameter:

$$\mu_{opt} = \frac{3D_m}{d_p} \quad \text{Eq. 3}$$

where D_m is the diffusion coefficient of an analyte in the mobile phase [20]. In conclusion, the analysis time is reduced with optimal flow rates for columns that are packed with smaller particles, while a higher pressure is needed to keep the optimal flow rate. The optimal flow rate for large molecules (with low D_m) are lower than for small molecules when small particles are used. An alternative to packed columns are monolithic columns, which will be described later.

4.3 Effect of downscaling chromatographic systems

It is important to be able to control the sensitivity parameters when the sample size is small and analyte concentration is low. By reducing the column ID, the analyte peak concentration in the detector becomes higher. In the column eluate, the maximum analyte concentration (C_{max}) is given by:

$$C_{max} = \frac{mN^{1/2}}{(2\pi)^{\frac{1}{2}}V_M(1+k)} \quad \text{Eq. 4}$$

where m is the mass of the analyte injected into the column, V_M is the void volume and k is the retention factor. V_M is a function of the column ID and the C_{max} ratio for two different columns will be the ratio of the squares of their corresponding ID values [21]. When injecting the same mass of sample into a column going from 1000 to 100 µm ID, the theoretical concentration gain at the detector would be 100 orders of magnitude for a concentration sensitive detector. ES-MS is a concentration sensitive detector over a comprehensive range of flow rates [22]. Therefore the use of reduced column ID may be used to achieve higher sensitivity. ES ionization can produce smaller droplets when the flow rates are low [23], see Figure 3, and when flow rates lower than 1 µL/min are used, the term nanoES or nanospray is used.

To achieve the best performance of miniaturized columns, dead volumes, before and after the column, must be reduced. "Zero dead volume" unions (PicoClear™ and peek tee) have been developed for this purpose [24] (Figure 4). These unions also ensure that there is no torsion of assembled capillaries which may produce particles that can clog the emitter [25].

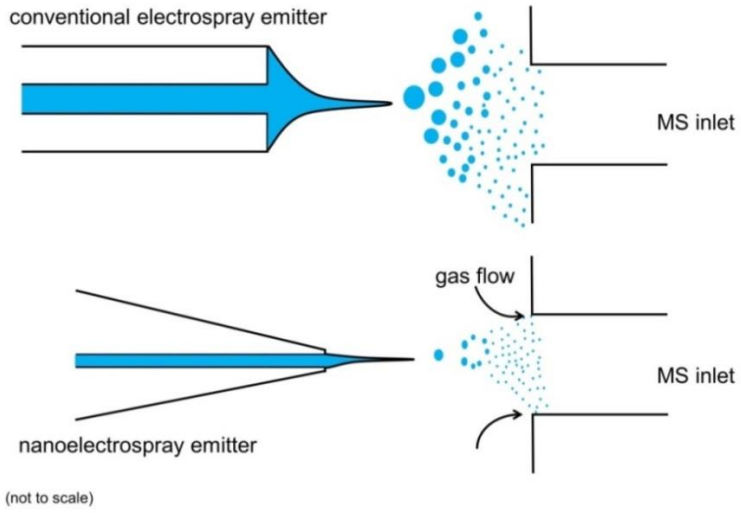

Figure 3: Comparison of high flow rates (top) and low flow rates (bottom) in ES. The low flow rate produces smaller droplets. Lower flow rates also allow closer proximity to the MS inlet, which affords more efficient ion introduction. In the illustration, a gas flow is shown to focus the small droplets into the MS. Figure adapted from [23].

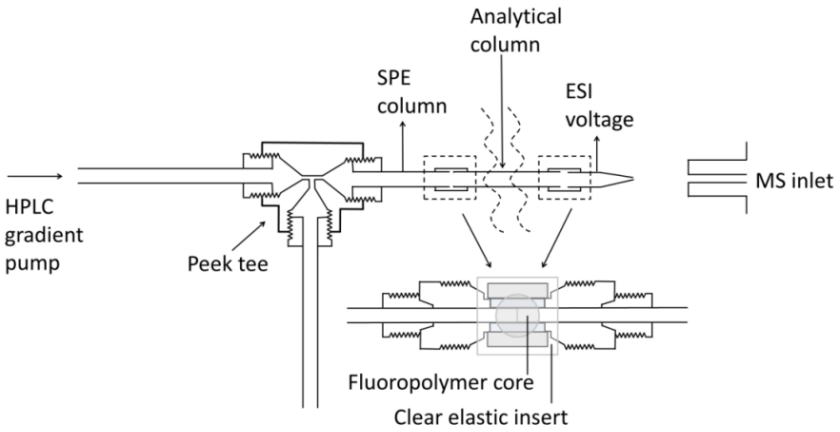

Figure 4: Illustration of "zero dead volume" unions used in a nano LC-MS platform. Fluoropolymer core (also known as PicoClear™) allow butt-to-butt connection between capillaries. Peek tee allows minimal dead volume when connecting three capillaries. Adapted from [24].

4.4 Column formats

The most common column format utilized in the analytical laboratories performing LC is still particle packed columns. For small sample sizes, narrow monolithic and open tubular (OT) columns (Figure 5) have shown promising performance [26-28], however these are not yet commonly used in routine laboratories.

In particle packed columns, the particles are enclosed in a (steel) housing by (steel) frits. Particle packed columns cannot be cut after the frit has been "installed" because then the particles would bleed out of the column. Monolithic columns, on the other hand, do not need frits, and the problem of bleeding of stationary phase is avoided. It consists of a single porous structure throughout the column. OT columns were originally developed for use in gas chromatography (GC) [29], but have also recently been successfully used in LC [26]. However, in order to achieve the same efficiency as packed columns, the ID of OT columns used in LC must be 10 µm or less [30].

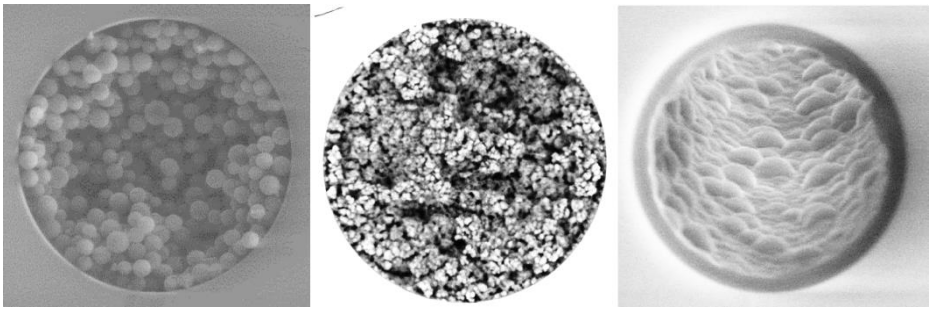

Figure 5: Scanning electron microscope (SEM) images of packed column (left), monolithic column (middle) and OT column (right).

4.4.1 Monolithic columns

Monolithic columns are a rather novel class of materials used in chromatography that emerged in the beginning of the 1990s [31]. Monolithic materials are polymerized by a mixture consisting of monomers, free-radical initiator, and porogenic solvent [32].

The resulting medium has the advantage of large through-pores that enable rapid flow-through, i.e. high permeability and low back-pressure [33]. This is partly due to the high porosity of monolithic columns compared to packed columns, which are 80 % and 40 % respectively. Another advantage of monolithic materials is the low mass transfer resistance.

The best way of improving mass transfer resistance in particle packed columns is by reducing the size of the particles and the diffusional path lengths in the pores. This improvement in particle packed columns results in less inter-particles void volume (V_M), but has the disadvantage of decreased permeability and increased back-pressure. Due to the rigid porous polymer throughout the monolithic column, these disadvantages are not present [34].

Monoliths have a very large surface area due to the tremendous amount of pore-channels in the polymer. The large surface area originate mostly from micropores, which have diameters of 2 nm and smaller, as well as mesopores ranging from 2-50 nm. The macropores, which are larger than 50 nm, does not contribute to the large surface area, but is essential in order to obtain the high throughput of liquid, and contributes to the low back-pressure [33].

Linear molecules exceeding 10^4 cannot penetrate the micropores. This makes the mesopores and macropores the only route for larger molecules. Hence, the pore size distribution is an important factor, and the mesopores provide most of the separation of large molecules [35].

There are two main types of monolithic materials, inorganic silica based and organic polymer based. Tanaka et al. introduced silica based monolith in 1993, and have since then successfully used these columns for separation of small molecules and peptides [36,37]. Organic polymer based monolithic columns, on the other hand, have been more used to separate large molecules such as proteins [34]. The inorganic monolithic columns have smaller pore sizes compared to organic monolithic columns.

PS-DVB and BuMa monolithic SPE columns have been prepared for this thesis.

4.4.2 PLOT columns

It is well known that 100-150 µm ID reversed phase particle packed capillary columns used in LC have the advantage of high resolving power, high sensitivity, and low sample and mobile phase consumption. However, analysis of samples of smaller size, such as cells from small tissue samples, can be problematic [38]. Therefore, more narrow-bore columns, as PLOT column, with an ID of 10 µm will offer reduced solute dilution in the column and hence improved sensitivity, and the mobile phase consumption may be reduced if pumps delivering low flow rate is available [26].

The success of using PLOT column in LC was limited [39,40] until Karger and his group successfully prepared a 10 µm ID PS-DVB PLOT column in 2007 [26]. Now, PLOT columns are also utilized for ultrasensitive LC proteomics analysis [26,28,41,42] and capillary electrochromatography (CEC) [43,44].

Karger's group developed a 10 µm ID PS-DVB PLOT column by using a similar procedure as for monolithic columns, except that the monomer solvent was substituted from a porogenic mixture to a single solvent [26]. This substitution (and space confinement) resulted in a polymer that precipitated at an earlier stage in the polymerization process [35,45]. The early precipitation formed a thin porous layer at the capillary wall, while center of the capillary tube remained open [26].

The PLOT column produced by Yue et al. [26] possessed high permeability that allowed the use of long column (4.2 m) by splitting the flow rate with the use of conventional HPLC pumps. The PS-DVB PLOT column demonstrated high efficiency, production reproducibility and good column-to-column retention time reproducibility. The 10 µm ID PS-DVB PLOT (Figure 6) column improved ES-MS sensitivity due to the increased analyte concentration eluted from the column combined with decreased ion suppression and enhanced ion collection efficiency at a low flow rate (20 nL/min). Later our group have used a 8 m long PLOT column with a 0.75 µm layer thickness [28]. Thinner film thickness enabled longer columns to be produced and used due to lower back-pressure compared to the 10 µm ID PS-DVB PLOT columns produced by Yue et al. [26].

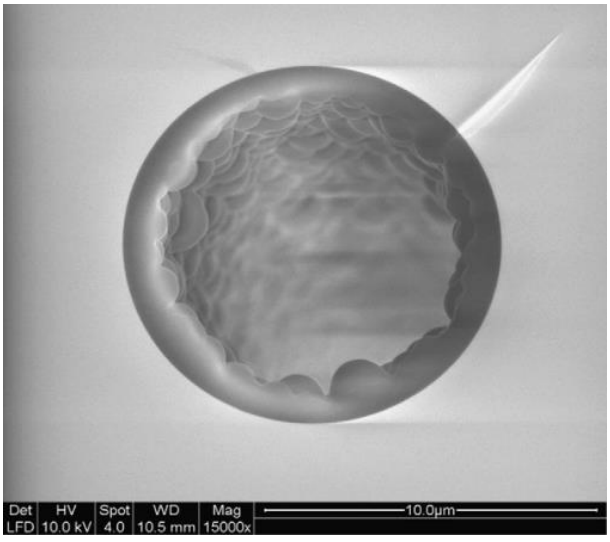

Figure 6: SEM image of PS-DVB PLOT column used for separation of intact proteins prepared by Rogeberg et al. [41].

4.5 Sample preparation in bottom-up proteomics: From proteins to peptides

The rate limiting step in bottom-up proteomic research is sample preparation, and existing protocols require at least overnight digestion (~16 h) [46]. The workflow is denaturation of the proteins, reduction and alkylation, before enzymatic digest of proteins into peptides. Protein digestion is the most time consuming step in sample preparation. Denaturation, reduction and alkylation

Before enzymatic cleavage of proteins to peptides, denaturation, reduction and alkylation of the proteins are necessary, see Table 2. This is in order to prepare the proteins for effective cleaving along the amino acid chains.

Table 2: The intended effect of denaturation, reduction and alkylation. Adapted from [47].

Procedure	Intended effect
Denaturation and reduction	Reduces disulphide bonds to uncoil the protein
Alkylation	Alkylation of SH groups, avoiding renaturation
Enzymatic digestion	Cleavage of proteins to peptides
Stop enzymatic digestion	To avoid autodigestion
Desalting and enrichment	Clean-up and enrichment of peptides

Denaturation and reduction of proteins break up the disulphide bonds in the proteins, and thereby unfold its tertiary structure. The reduction of disulphide bonds to thiols can be initiated by a combination of heat and a reaction with chemicals containing sulphydryl or phosphine groups such as 1,4-dithiothreitol (DTT) [48], β-mercaptoethanol [49] or tris(2-carboxyethyl)phosphine [50]. DTT is the most common chemical used for reduction of disulphide bonds, because it is a strong reducing agent. The free sulphydryl groups are highly reactive and will oxidize with other sulphydryl groups. For this reason, sulphydryls are blocked by alkylation to prevent unwanted reactions [50], such as inter- and intra-molecular disulphide formation between cysteines in the protein. Reduction of the disulphide bonds, or renaturation of proteins, can be avoided by combining denaturation and reduction, see Figure 7.

Alkylation of cysteine is performed after denaturation and reduction. Alkylation of cysteine is done in order to reduce the potential renaturation, see Figure 7. Iodoacetamide (IAM) [51] and iodoacetic acid (IAA) [52] are the most common agents for alkylation.

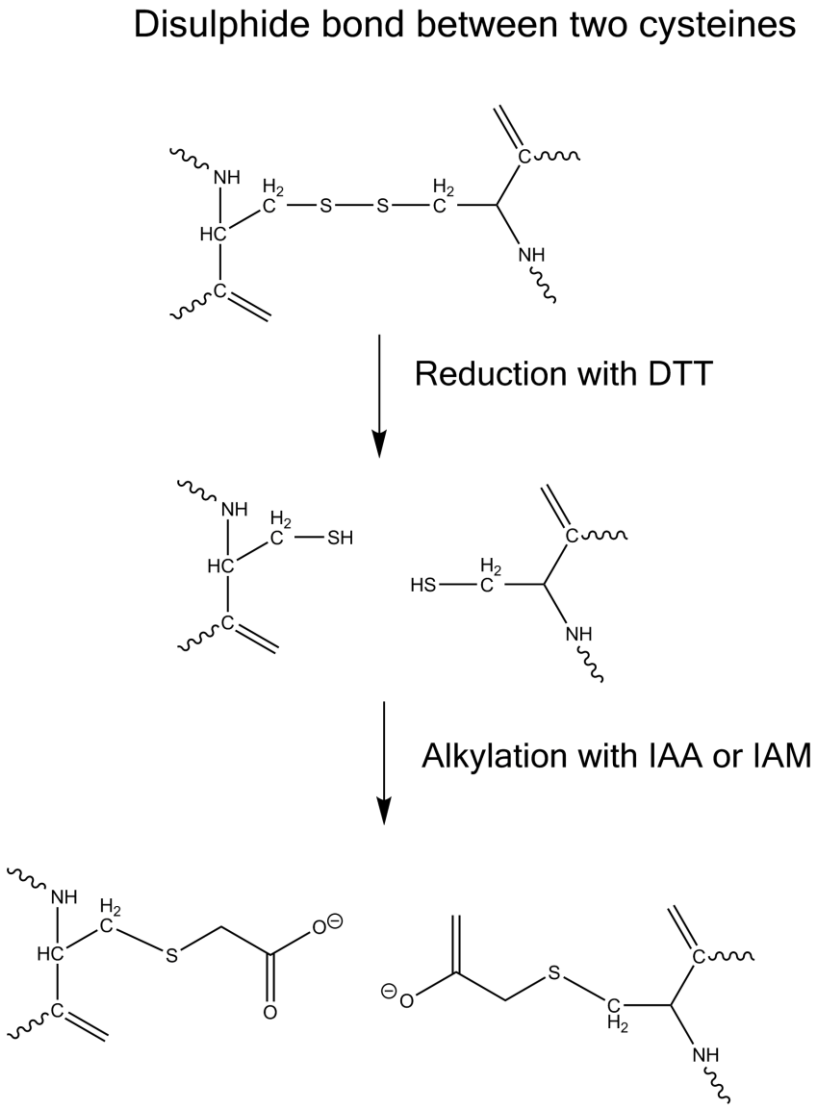

Figure 7: Reaction chemistry of reduction with DTT and alkylation, in this case, with IAA. Adapted from [53].

4.5.1 Protein digestion

The next step after denaturation, reduction and alkylation, is digestion of the proteins into peptides. The most common digestion agent is trypsin, which is a serine protease that specifically cleaves proteins on the carboxyl side of the amino acids lysine (Lys, K) and arginine (Arg, R) [54]. Trypsin hydrolyses the peptide bonds after Lys or Arg residue unless they are N-linked to aspartic acid (Asp), or followed by proline (Pro) on the carboxyl side [47]. The resulting peptides are in the preferred mass range for MS sequencing. The cleavage of proteins to peptides results in information-rich peptide fragmentation spectra that are easy to interpret.

Another serine protease, which is also quite commonly used in proteomics, is Lys-C. It is active in an harsh environment with 8 M urea, and gives larger fragments than trypsin as it only hydrolyses at the carboxyl side of the amino acid Lys [54]. Lys-C originates from the bacterium *Lysobacter enzymogenes* [55].

The most common temperature during in-solution digestion is 37 °C [47]. The optimal pH for tryptic digestion is between 7.0 and 9.0 [56]. In order to achieve the ideal pH interval 50 mM triethyl ammonium bicarbonate (tABC) or 12.5 mM ammonium bicarbonate (ABC) buffer [51] is added. Capelo et al. reports that elevated temperature could speed up the digestion [57]. Complete digestion was achieved in 1 h at 60 °C vs. 12 h at 37 °C, while Turapov et al. concluded that a temperature gradient compared to the conventional procedure gives a better score when searching for the peptides in a database [58].

When performing in-solution digestion, the enzyme to substrate (protein) ratio is an important factor to ensure that there is sufficient amount of enzyme to perform the digestion. If the substrate to enzyme ratio is too high, then autolysis, or autodigestion, can occur. Hustoft et al. [47] reported that a sufficient enzyme to substrate ratio (E+S) is 1+20.

The ideal digestion time when performing targeted proteomics may vary, and a study to find the optimum in-solution digestion time should be performed. When dealing with comprehensive proteomics, digestion time up to 9 h is recommended by Proc et al. [59]. Conventional in-solution digestions have been reported using digestion times from 12-16 h, but 24 h have also been reported [51]. Therefore, a more convenient digestion time would be overnight and the post sample digestion steps could be continued the following day.

2-step mechanism for protein digestion

As mentioned above, trypsin and Lys-C are examples of serine proteases. The principle of how these enzymes cleave the amino acid chain is similar, namely through the accepted 2-step hydrolysis reaction that is shown in Figure 8.

More peptides are expected to be generated when digestion is performed by trypsin compared to Lys-C, because trypsin cleaves at more sites compared to Lys-C. However, less missed cleavages are expected after cleavage with Lys-C [13].

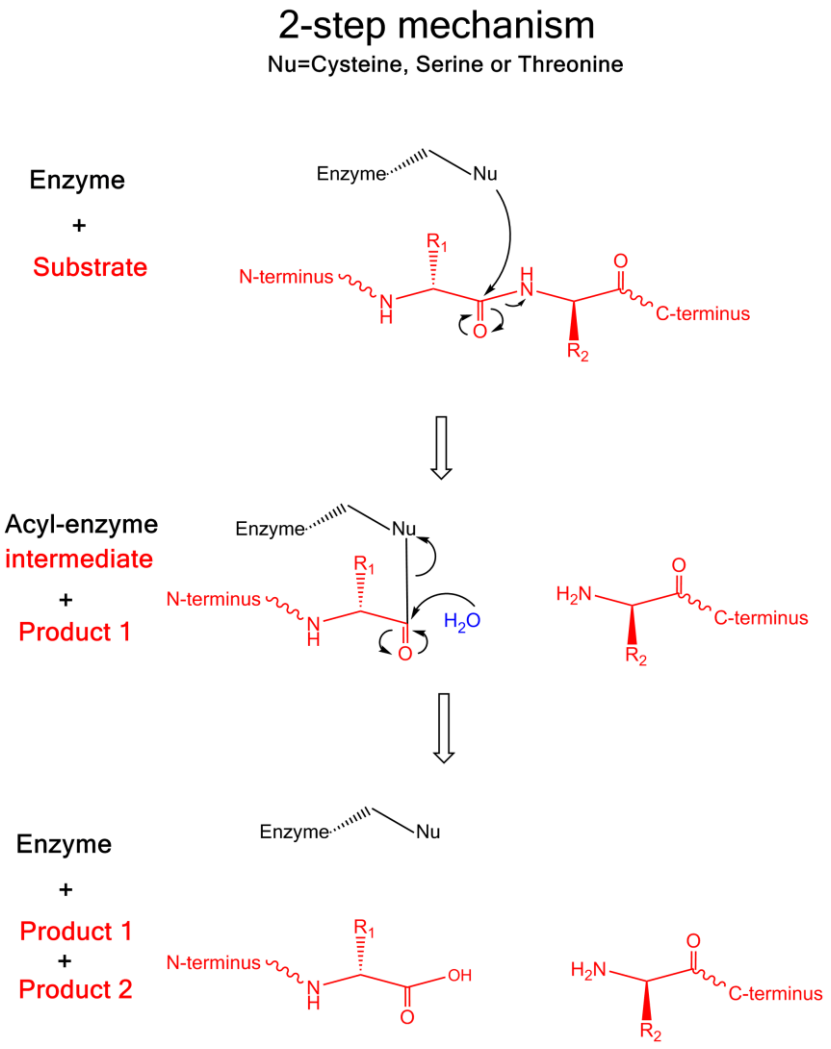

Figure 8: Catalytic mechanism of proteolysis. The enzyme is active and acts as a nucleophile (Nu) as it combines with the amino acid chain of the protein. A covalently link between the N-terminal half of the protein and the enzyme is formed, before water hydrolyses this intermediate and completes the catalysis.

4.5.2 Detection

The resulting peptides are commonly separated by reversed phase LC and detected by ES-MS. When peptides are detected by the MS, very sophisticated algorithms are necessary in order to find the matching proteins. Examples of such algorithms are Mascot and Sequest [60]. Proteome Discover is a program that uses these algorithms in order to map the peptides in the amino acid sequence of the protein, and delivers an amino acid SQ %. This can be used to measure the efficiency of the digestion.

As mentioned above, in-solution digestion is very time consuming, and there are possibilities of contaminations. Keratins are proteins from fingerprints, hair, wool clothing, latex gloves, dust, and skin flakes [61]. Keratin contamination can occur during in-solution digestion due to the manual handling. If the concentration of keratin is greater than that of the protein of interest, then the corresponding peptides can be disguised by keratin in the LC-MS-system. The MS will select the keratin peptides instead of those of the protein of interest for MS/MS. This would result in little or no information about the actual protein [47]. The digestion can be performed in a chromatographic LC-MS-system (i.e. on-line) in order to decrease the number of manual steps during protein digestion in the sample preparation workflow. On-line digestion could also decrease keratin contaminations and more MS/MS information from the specific protein could be extracted.

4.5.3 Accelerated protein digestion: Immobilized enzyme reactor (IMER)

As mentioned above, in-solution digestion may result in contaminations, and the digestion time is long. In order to obtain low detection limits in targeted proteomics and to identify many proteins in comprehensive proteomics, there is a need for an efficient and fast proteolytic digestion. Capelo et al. have summarized ways to accelerate protein digestion [57]. Among these are heating, microspin columns, ultrasonic energy, high pressure, infrared and microwave energy, alternating electric fields and IMER.

IMER dates back to the 1950s according to Ma et al. [62]. Today, enzymes have been immobilized onto inorganic particulate materials [63], inorganic monolithic materials [64,65], organic monolithic materials [66,67] and organic membrane materials [68]. Trypsin has been immobilized onto OT [69] and monolithic [70] stationary phases, and our group has recently

optimized an OTER, which can digest protein samples in 30 min [27], and developed an OTER that can digest complex samples [71].

In-solution digestion may have, as mentioned earlier, the unwanted formation of peptides caused by enzyme autodigestion. Enzyme autodigestion can lead to ion suppression in the MS analysis, and this will make the interpretation of the data more complicated [72]. Other drawbacks with in-solution digestion are low efficiency, extended incubation time and manual sample manipulation [73]. A great advantage of IMER is that they can be coupled on-line with an LC-MS-system. IMERs are possible to reuse and they constitute a large enzyme to substrate ratio [74]. Immobilized trypsin in microreactors minimizes protease autolysis, achieves high digestion efficiency and reduces digestion time [75]. For example, digestion of the proteins bovine serum albumin, β-casein, cytochrome C (Cyt C) and phosphorylase b could be performed in 20 s in an open-channel microchip [76,77]. Another group, which immobilized pepsin covalently into a dextran particle-based capillary, could report digestion of bovine serum albumin, myoglobin (Myo) and human hemoglobin (Hemo) within 3 min [78]. In addition to that, an open-channel microreactor immobilized with trypsin onto a sol-gel matrix material digested β-casein, Myo and Cyt C within impressive 24 s [79].

4.6 Preparation of OTER, monolithic SPE and PLOT columns

The preparation of SPE and PLOT columns for miniaturized LC is performed in fused silica capillaries and consists of three steps, pre-treatment, silanization and polymerization. Preparation of OTERs requires an extra step called immobilization.

4.6.1 Pre-treatment

Hydrolyzing the inner wall of fused silica capillaries prepares the capillary for silanization, and removes any contaminations that arise from the process of making fused silica. The siloxane bonds on the wall of fused silica are hydrolyzed to silanol groups with a strong base (Figure 9a) in order to make them reactive towards the silanization reactant γ-(trimethoxysilyl)propyl methacrylate (γ-MAPS). A review by Courtois et al. [80] compares

different pre-treatment procedures, and it concludes that a longer pre-treatment step at elevated temperatures resulted in a higher percentage of silanol groups at the surface.

4.6.2 Silanization

Silanization, sometimes called vinylation, is the process of attaching a reactive group (serving as an anchoring site) at the surface of the fused silica. A monomolecular layer of reactive chain molecules is covalently attached by siloxane linkages to the pre-treated capillary wall (Figure 9b). The most common silanization agent, γ-MAPS, reacts with the silanols at the surface favorably at elevated temperatures [81,82]. The reagent will auto-polymerize at high temperatures, and therefore an inhibitor, 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH), is added to the mixture [83]. The silanization process is often carried out at 110 °C, as performed in [26], however, other temperatures and silanization times have been reported [84-86].

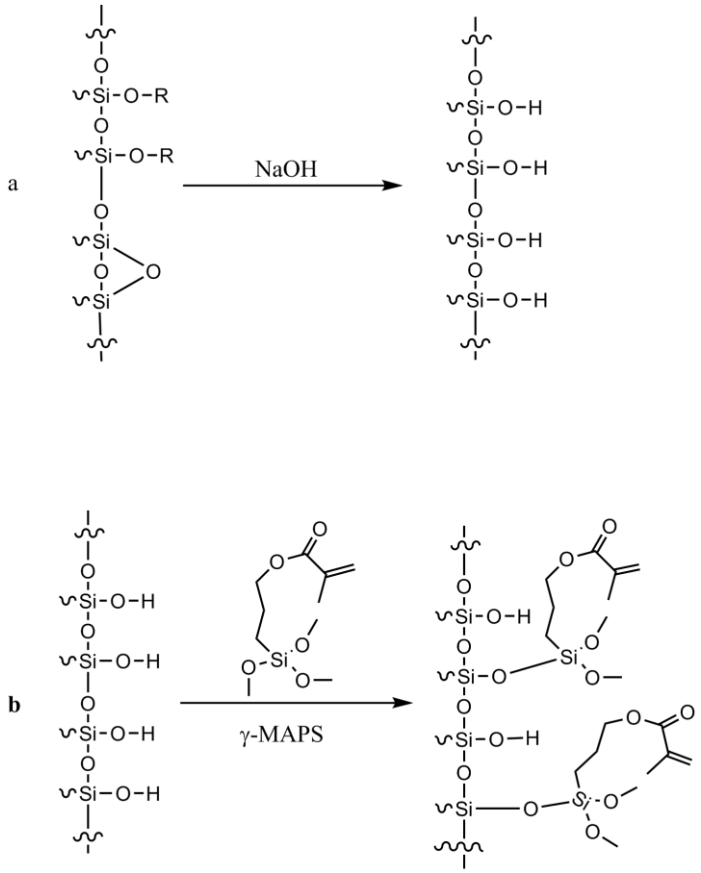

Figure 9: a) The reaction for the pre-treatment of capillary. b) Silanization reaction of the pre-treated capillary.

4.6.3 Polymerization

General for all columns prepared in this study, a solution containing a combination of monomers, radical initiator, and a porogenic mixture of solvents is filled into the silanized capillary [26]. For monolithic columns, one of the monomers must be a crosslinker in order to create the network of polymer. The radical initiator, AIBN, triggers the polymerization under presence of Ultraviolet (UV)-light [87], heat between 55-80 $^{\circ}$C [33,88], or redox reaction [89]. Triggering the polymerization by UV-light has the advantage of fast polymerization, but the disadvantage are the need of transparent tubing and a strong UV-source [90]. Figure 10a shows the reaction mechanism for polymerization of methacrylate monomers, and Figure 10b shows cross-linking for porous polymeric structure. Heat was used in this study to initiate the polymerization reactions.

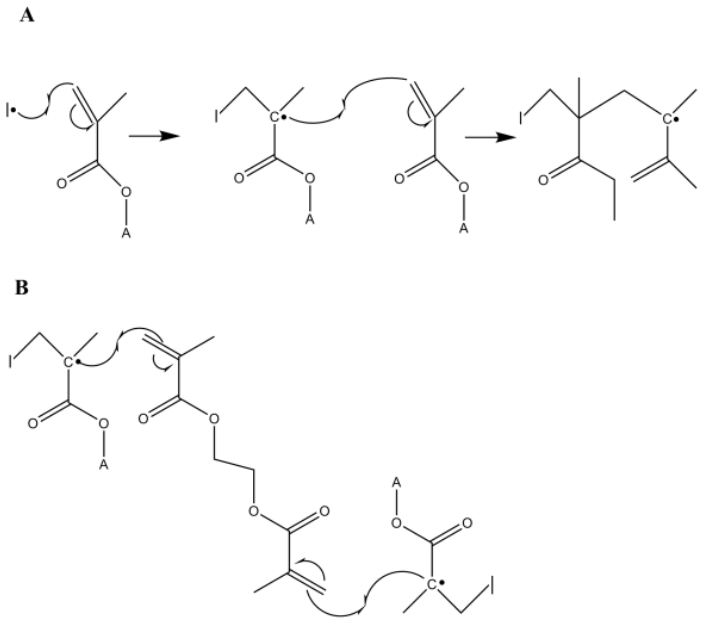

Figure 10: a) Growing polymer chain caused by radical initiation of the monomer. b) Radical polymerization. Figure adapted from [88].

The porogen is a pore-forming solvent, and it is the most used parameter to control the porosity without changing the chemical composition of the polymer. At the beginning of the polymerization process, the porogen controls the porous properties by solvation of the polymer chains in the medium where the reaction takes place [33]. Temperature, porogenic solvent and amount of crosslinker have been shown by Viklund et al. to affect the pore size distribution [35].

The nucleation rate, i.e. the rate of forming a nucleus, increases with increasing temperature. This leads to larger pores. More and smaller globules are formed when the amount of monomer is the same [35]. The polymer phase is not soluble in the polymerization solution and will precipitate as the polymerization reaction proceeds. It is not soluble because the molar mass (Mm) exceeds the solubility limit of the polymerization solution, or it might be insoluble due to cross-linking [35]. Larger pores are achieved if the monomers are solved in a poor solvent, because the polymers will precipitate earlier form the solution. In addition to that, the polymerization reaction continues in the larger polymer globules. The voids between the globules, called pores, grow larger as the globules become larger. The globules and pores become smaller when increasing the amount of cross-linking monomer in the monolithic polymerization solution, because it leads to faster precipitation. It has also been reported that the final monolith is affected by the amount of crosslinker added [35].

Another factor affecting the polymerization, investigated by Nischang et al., is the amount of γ-MAPS at the surface of the silanized capillary [91]. This was investigated by varying the silanization time of the wall surface in a 10 μm ID capillary. No anchoring sites for the polymer were present when no γ-MAPS were at the surface. However, increasing the silanization time from 5 min to 2 h showed that the polymer was successfully anchored to the wall of the capillary, see Figure 11.

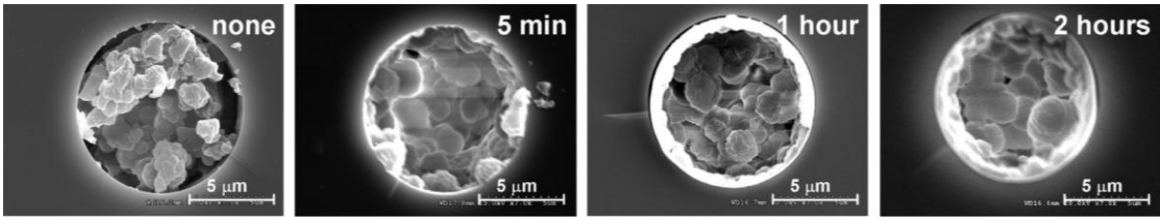

Figure 11: The effect of silanization time on the polymer anchored to the walls of the capillary. Figure adapted from [91].

The difference in column preparation from monolithic to OT columns is that the solvent used to solve the monomers is changed from a porogenic mixture to a single solvent [25]. This gives a precipitation of the polymer at an earlier stage during the polymerization process [45,80], which forms a thin layer at the inside of the capillary wall, while the center of the capillary is open [26].

4.6.4 Enzyme immobilization into OTERs

OTERs are polymerized as OT columns before enzyme immobilization, which is performed through adsorption, encapsulation or entrapment [92]. Immobilization by covalent attachment of enzyme is the most common. One example of covalent immobilization of enzymes onto polymer is through azlactone functionalities.

4.7 Aim of study

The aim of this study was to develop suitable enzyme reactors for protein digestion in an automated nanoproteomic platform employing narrow ID monolithic SPE, for trapping, and PLOT "in-lab-made" columns for separation of peptides.

5 Experimental

5.1 Materials and reagents

HPLC grade ACN (VWR, West Chester, PA, USA), HPLC water (Chromasolv plus for HPLC, Sigma Aldrich, St. Louis, MO, USA), type 1 water from an ultrapure water purification system (Millipore Corporation, Billercia, MA, USA), formic acid (FA, 50 %, Fluka, by Sigma Aldrich), NH_4OAc (98 %, Sigma Aldrich), ammonia (28 %, VWR Fontenay-sous-Bois, France) were used to prepare the mobile phases. All proteins were reduced and alkylated prior to an on-line digestion in the proteomic platform with tABC (pH: 8.5±0.1, Sigma-Aldrich), DTT (Fluka, Sigma Aldrich), IAM (Sigma Aldrich). DTT and IAM were dissolved in 20 mM phosphate buffer. Sodium phosphate monobasic (99 %, Sigma Aldrich) was used to prepare the phosphate buffer. Trifluoroacetic acid (TFA) terminated the digestion, and was purchased from Sigma Aldrich. The standard proteins used were Cyt C (bos taurus, 11.7 kilo Daltons (kDa)), Hemo (Homo sapiens, 15.2 kDa), Myo (equine heart, 17 kDa), CaA (carbonic anhydrase 2, bos taurus, 29.1 kDa), immunoglobulin G (IgG, Homo sapiens, 36.1 kDa), human serum albumin (HSA, Homo sapiens, 69.3 kDa), transferrin (Transf, Homo sapiens, 77 kDa) and fibrinogen (Fibr, bos taurus, 340 kDa). These proteins were obtained from Sigma-Aldrich, while beta-catenin (β-cat, Homo sapiens, 85.4 kDa) was obtained from Millipore Corporation. ProGRP was obtained as described by Torsetnes et al. [93].

For preparation of the columns the following reagents were used: N,N-dimethylformamide anhydrous (DMF), 3-(trimethoxysilyl)propyl methacrylate (γ-MAPS, 98 %), divinylbenzene (DVB, 80 % mixture of isomers), styrene (99 %), 1-dodecanol, 1-heptanol, 1-decanol (98 %), sodium hydroxide (NaOH, 99 %), inhibitor DPPH, EDMA (ethylene dimethacrylate, 98 %), HEMA (97 %, containing 200-220 ppm monomethyl ether hydroquinone as inhibitor) and initiator AIBN, all purchased from Sigma Aldrich. VDM was purchased from Polysciences, Inc. (Warrington, PA, USA). Toluene was purchased from Rathburn Chemical Ltd. (Walkerburn, Scotland, UK). The porogen 1-cyclohexane-dimethanol was obtained from The Dow Chemical Company (MI, USA). Ethanol (EtOH) was purchased from Arcus (Oslo, Norway). Trypsin from bovine pancreas (≥ 10,000 BAEE), benzamidine (>95 %), and ethanolamine (99 %) were all purchased from Sigma Aldrich. Lys-C and T/L mixture were purchased from Promega (through Nerliens Meszansky (Oslo, Norway)). Ethanolamine was

used to quench the unreacted sites on the polymer and was obtained from Sigma Aldrich. N_2 (99.99 %) was obtained from AGA (Oslo, Norway). Polyimide coated fused silica tubing (360 µm outer diameter (OD), 100, 75, 50, 30, 20, 15, 10 and 5 µm ID) were purchased from Polymicro Technologies (Phoenix, AZ).

5.2 Column preparation

The columns were prepared by using an in-house made pressure bomb (Figure 12). The pressure bomb was used to fill the capillaries during the pre-treatment, silanization, polymerization and immobilization steps. It was also used for rinsing of the column with ACN, H_2O, and N_2. The pressure inside the bomb could be varied to a pressure that gave a feasible flow rate through the capillary.

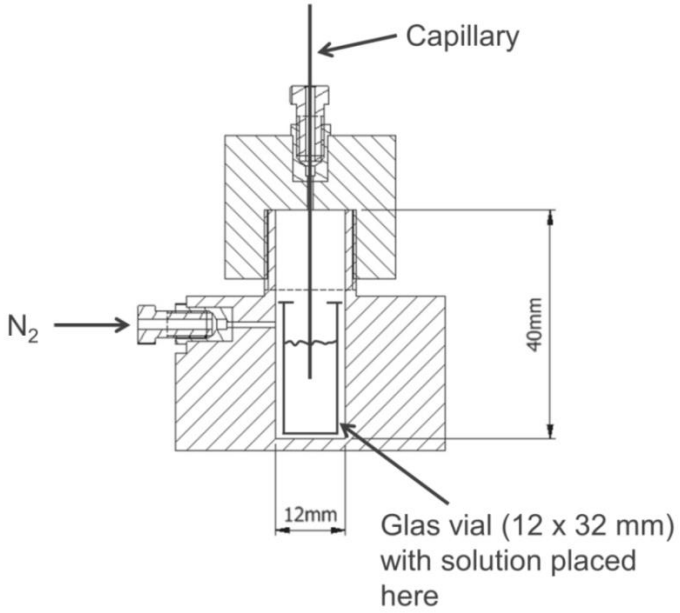

Figure 12: In-house made pressure bomb used to prepare the columns. Adapted from Inge Mikalsen.

The following procedure for pre-treatment and silanization was carried out for all the columns prepared during this study. A weighed amount of reagent was measured by using a micropipette and a glass vial (placed on a Mettler AE 166 balance). The pressure bomb was used to transfer the solutions into the capillaries. The nitrogen flask produced a pressure

ranging from 100-200 bar inside the pressure bomb. Fused silica capillaries were cut in the desired length by a capillary cutter, and were filled with 1M NaOH in order to activate the silanol groups, making them able to create a bond with the silanization mixture. After filling of the capillary, the ends were plugged with a GC septum. The capillaries filled with NaOH solution were placed in a GC oven (GC 8035, Fisons instruments, Ipswich, UK) at 100 °C for 2 h. Then they were flushed with water for 30 min followed by flushing with ACN for 30 min, and finally dried with N_2 to remove any liquid from the capillaries. The silanization solution, consisting of 0.3135 g γ-MAPS and 0.0050 g DPPH in 0.6608 g DMF, was homogenized in an ultrasonic bath and filled into the capillaries by the pressure bomb. The capillaries were subsequently sealed in both ends by a GC septum and placed in the oven at 110 °C for 6 h. The capillaries were then flushed with ACN for 30 min and dried with N_2 for 30 min before proceeding with the polymerization step. For "know-hows" regarding column production, see section 9.4 in Appendix.

5.2.1 PS-DVB PLOT column preparation

The PS-DVB PLOT column was prepared as described by Yue et al. [26] with a few modifications and minor adjustments as reported by Rogeberg et al. [28]. The polymerization solution consisted of 0.0050 g AIBN, 0.1818 g styrene, 0.1828 g DVB and 0.7434 g EtOH (~70 % v/v). The polymerization solution was placed in an ultrasonic bath for 5 min for degassing and homogenization. A 10 µm ID silanized capillary was filled with the polymerization solution before both ends were plugged with a GC septum and placed in the oven at 74 °C for 16 h (Figure 13). The reaction chemistry for the preparation of PS-DVB PLOT column is shown in Figure 14.

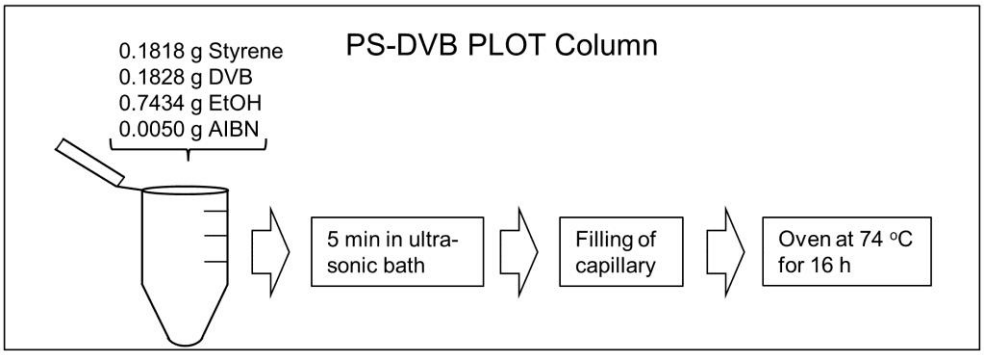

Figure 13: Step-by-step illustration of how 10 µm ID PS-DVB PLOT columns were prepared.

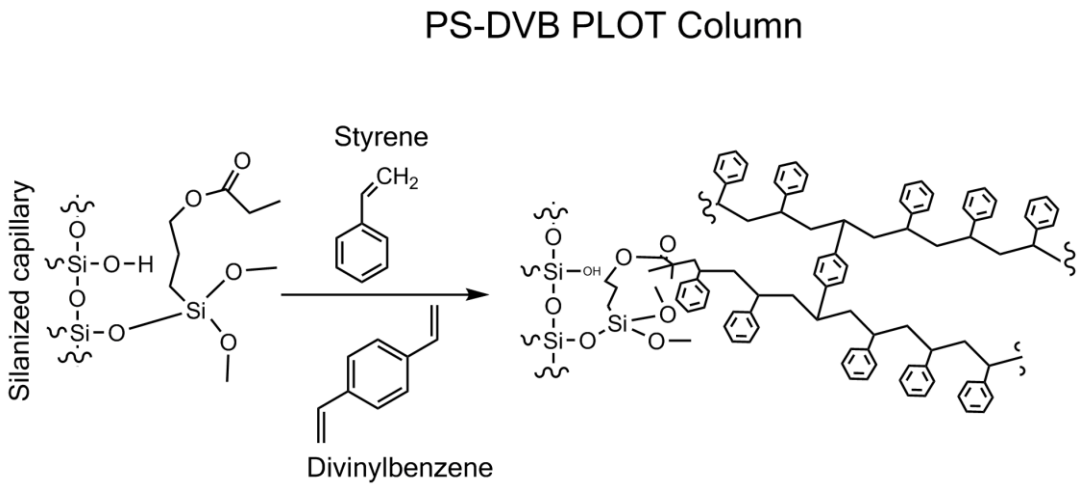

Figure 14: Reaction chemistry going from silanized capillary to polymerized PS-DVB PLOT columns.

After the polymerization step, the column was washed with ACN for 30 min, and dried with N_2 for 30 min as the column was to be studied with the use of microscopes (see section 5.5 for more information).

Typically, a couple of cm of the column had to be cut at both ends in order to see the polymer layer in the microscope. The lengths of the capillaries throughout the column preparation steps when making a 100 cm PS-DVB PLOT column are shown in Figure 15. The reduction of the length of the capillaries throughout the process is due to necessary trimming of the capillary when inserting it into the pressure bomb each time. Trimming is also done because the capillary scratches the inside of the graphite ferrule when inserting the capillary into the pressure bomb which may cause particulates to enter the capillary inlet and cause clogging.

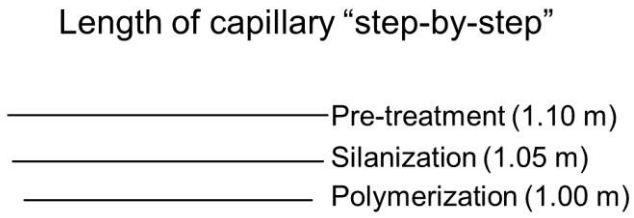

Figure 15: Schematic illustration of the length of capillary throughout the preparation steps of 10 µm ID x 100 cm PS-DVB PLOT columns. Not to scale.

5.2.2 Preparation of PS-DVB monolithic SPE columns

The 50 µm ID PS-DVB monolithic column was used as a SPE column, and its preparation was based on a paper by Lv et al. [94].

The polymerization solution was prepared by weighing out and mixing 0.2100 g styrene, 0.1300 g toluene, 0.1900 g DVB, 0.4700 1-decanol, and 0.0040 g AIBN into a glass vial (Figure 16). The mixture was placed in an ultrasonic bath for 5 min before it was filled into a pretreated and silanized 50 µm ID capillary with the pressure bomb. After filling of the polymerization reaction, the capillary was sealed by a GC septum. The polymerization reaction (Figure 17) was initiated by placing the capillary in an oven at 70 °C for 20 h After polymerization, the column was rinsed with ACN for 30 min and dried with N_2 for 1 h. The lengths of the capillary throughout the process of making the monolithic column are shown in Figure 19. The length of the monolithic column is usually about 20 cm after polymerization.

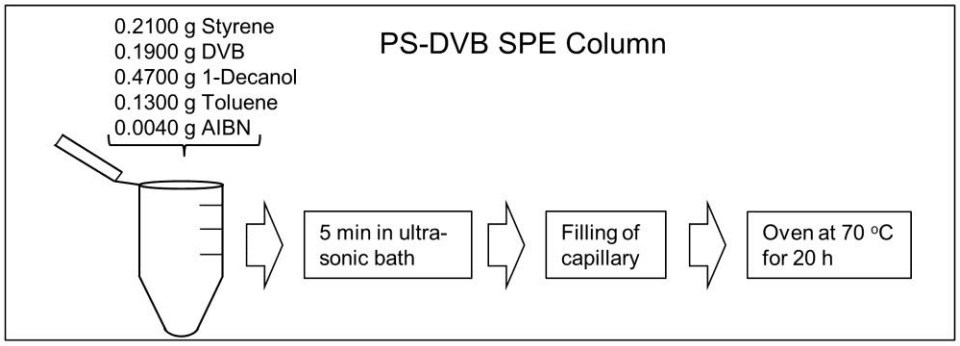

Figure 16: Illustration of the preparation of 50 µm ID PS-DVB monolithic SPE column.

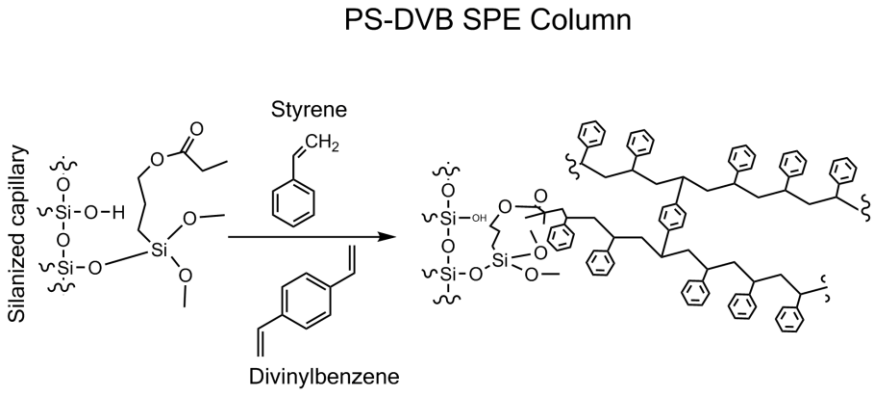

Figure 17: Reaction chemistry going from silanized capillary to polymerized PS-DVB SPE column.

5.2.3 Preparation of BuMa monolithic SPE columns

The 50 µm ID x 4.5 cm BuMa SPE column was based on the recipe by Geiser et al. [95] and also used in a previous published paper by Rogeberg et al. [28].

The polymerization solution consisted of 0.2400 g BuMa, 0.3400 g 1-propanol, 0.2600 g 1,4-butanediol, 0.1600 g EDMA and 0.0040 g AIBN. The polymerization solution was sonicated for 5 min before it was filled into the pre-treated and silanized 50 µm ID capillary. The polymerization took place in an oven at 70 °C for 16 h (Figure 18).

When the polymerization was finished, the column was removed from the oven and flushed with ACN for 30 min before it was dried with N_2 for 1 h. The length of the capillary throughout the process of making the BuMa monolithic SPE column is shown in Figure 19.

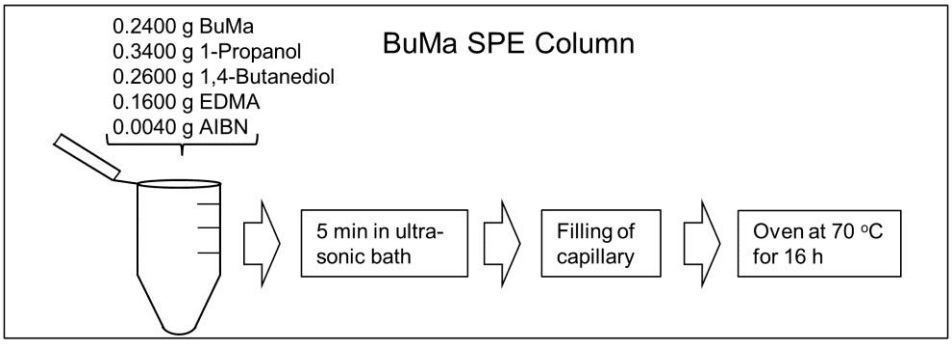

Figure 18: Schematic drawing of how the 50 µm ID BuMa monolithic SPE columns were prepared.

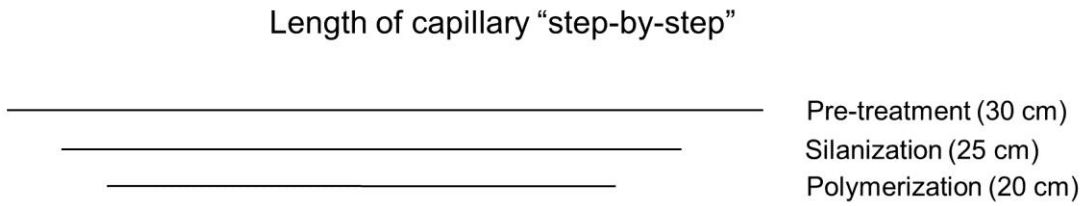

Figure 19: Schematic illustration of the length of the capillaries throughout the preparation steps of 50 µm ID x 20 cm PS-DVB and 50 µm ID x 20 cm BuMa monolithic SPE columns.

5.2.4 Preparation of OTERs

All OTERs were prepared in 20 µm ID capillaries. In addition, a 50 µm ID capillary was used in an attempt of making a monolithic HEMA-VDM reactor (see Appendix in section 10).

Polymerization

Two polymerization solutions were used. The polymerization solution for capillaries up to 30 cm consisted of 0.0800 g HEMA, 0.0200 g VDM, 0.6000 g 1-decanol and 0.0001 g AIBN. For OTERs up to 1.5 m, 1-decanol was replaced by 1-heptanol.

A 20 µm ID capillary was filled with the polymerization solution using the pressure bomb, and sealed with a GC septum. The polymerization was performed in a GC oven with a temperature program (65 °C for 5 h, followed by 80 °C for 5 h). The polymerization was initiated right after the filling of the capillary, in order to avoid precipitation of AIBN, which could lead to uneven polymerization. After the polymerization reaction was complete, the GC oven was programmed to room temperature (25 °C) until the capillary was removed the next morning and dried with N_2 for 30 min.

The general description of how OTERs were prepared is also presented in Figure 20, while the chemical reactions for the preparation of these columns are shown in Figure 21. The length of the capillary during the preparation of OTERs is presented in Figure 22.

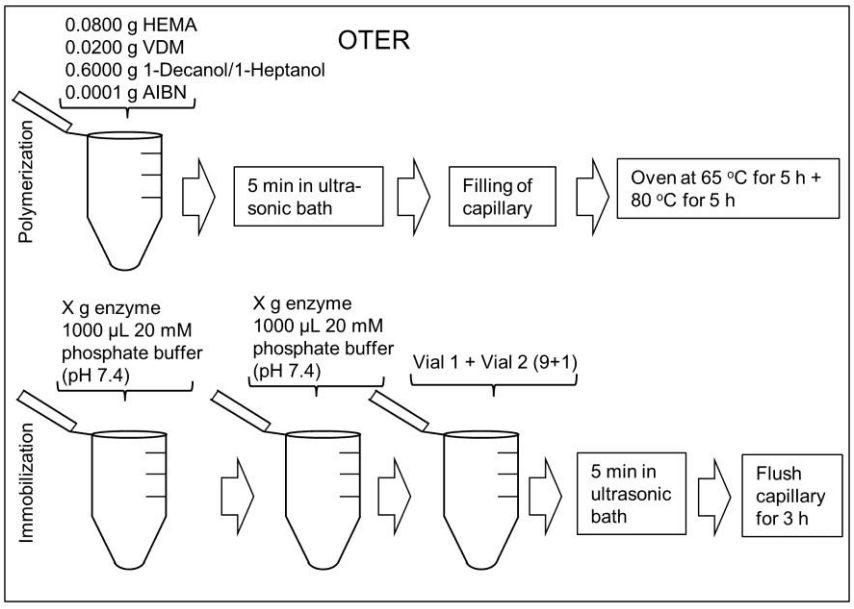

Figure 20: Step-by-step illustration of 20 µm ID OTER preparation.

Immobilization

The procedure for immobilization of trypsin, Lys-C and T/L in the HEMA-VDM polymerized OT columns was the same, but with different initial concentrations (Table 3).

Table 3: Initial concentration of trypsin, Lys-C and T/L used for immobilization on HEMA-VDM OT columns.

	Initial Concentration
Trypsin	2.5 mg/mL
Lys-C	15 µg/mL
T/L	20 µg/mL

As an example, trypsin immobilization was performed by flushing the column with a trypsin-solution for 3 h at a pressure between 120-200 bar.

The trypsin-solution consisted of 900 µL from vial A and 100 µL from vial B, where vial A consisted of 2.5 mg trypsin dissolved in 1 mL of 20 mM phosphate buffer with a pH of 7.4, and vial B consisted of 2.5 mg benzamidine dissolved in 1 mL of 20 mM phosphate buffer with a pH of 7.4. After immobilization, the column was subsequently filled with 50 mM NH_4OAc and stored at 4 $^{\circ}C$.

The first OTERs were quenched by flushing the columns with a 1 M ethanolamine solution for 1 h at 120-200 bar. However, when longer OTERs were developed, quenching did not show any effect, and therefore this was left out of the OTER procedure.

A video showing the preparation of OTERs has been made for this study, and can be seen at http://studio.usit.uio.no/kjemi/OTER.mov (also used in manuscript by Hustoft et al. [71]).

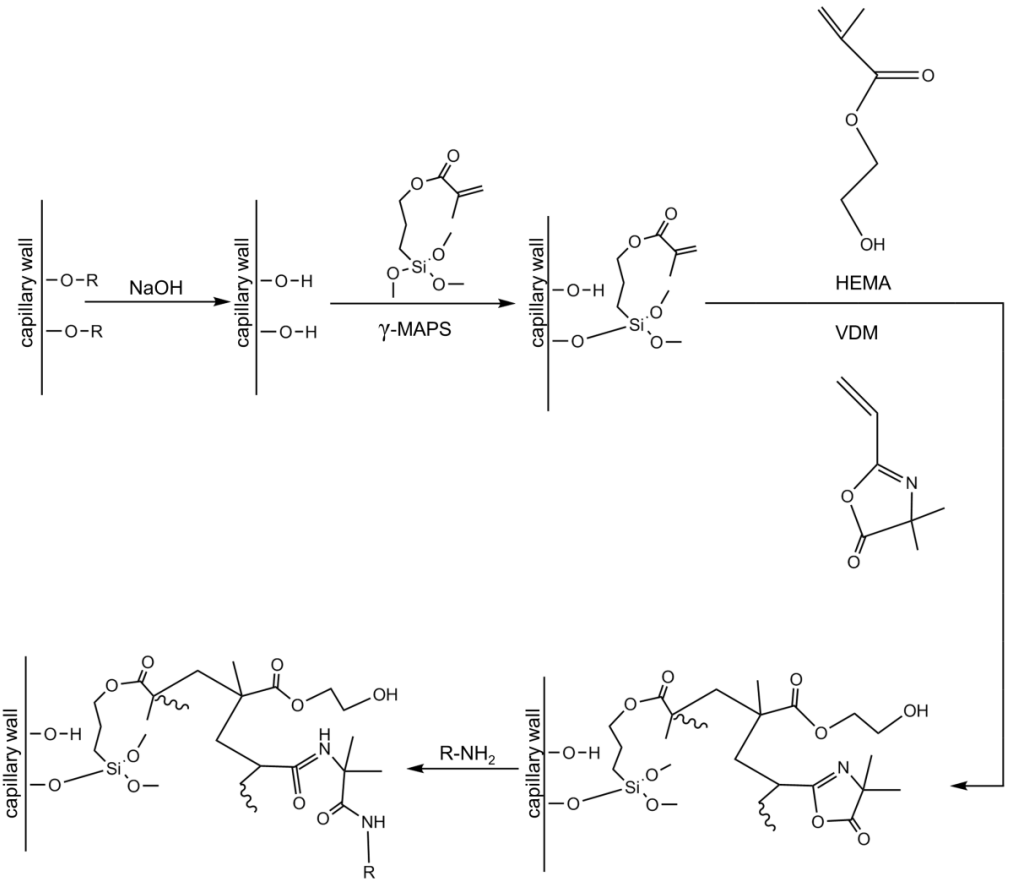

Figure 21: Reaction chemistry of the preparation of OTERs. Pre-treatment, silanization and polymerization followed by immobilization with enzyme (R = trypsin, Lys-C or T/L) is shown.

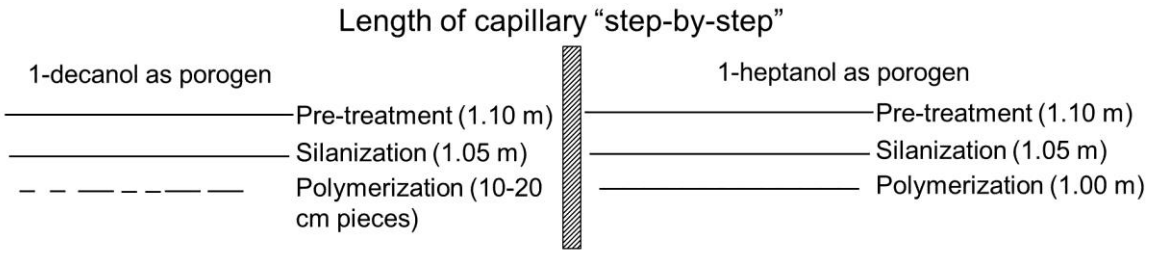

Figure 22: Schematic illustration of the length of capillary throughout the preparation steps of 20 µm ID x 10-20 cm OTERs (prepared by 1-decanol as porogen) and 20 µm ID x 100 cm OTERs (prepared by 1-heptanol as porogen). Not to scale.

5.3 Protein standard solutions and samples

5.3.1 Proteins used for working solutions

The proteins used for evaluation of the OTER in the LC-MS-system are listed in Table 4. Some of the initial experiments were carried out with in-solution digested protein solutions (see section 5.3.5). The size of the proteins varied from 12 to 340 kDa. A working solution consisting of 5 µg/mL of each protein was made by diluting 5 µL of 1 mg/mL protein in tris-HCl pH 8.0 in a Protein LoBind Eppendorf vial.

Table 4: Mm of each protein, in ascending order, used for preparing the protein standard solutions.

Protein	Abbreviation	Mm (kDa)
Cytochrome *C*, bovine	Cyt C	11.7
Hemoglobin, bovine	Hemo	15.2
progastrin-releasing peptide isoform 1	ProGRP	16.2
Myoglobin	Myo	17.1
Carbonic anhydrase 2, bovine	CaA	29.1
Immunoglobulin G, human	IgG	36.1
Human serum albumin	HSA	69.3
Transferrin, human	Transf	77.0
β-catenin	β-cat	85.4
Fibrinogen, bovine	Fibr	340.0

5.3.2 Protein working solutions

Protein stock solutions were prepared by dissolving 1 mg protein in 1 mL H_2O (1 mg/mL protein). These were stored at -18 $^{\circ}$C until use.

Two working solutions containing all 10 proteins were prepared. One protein standard solution (Mix I) containing 5 µg of each protein was dissolved in 1 mL of 50 mM tABC buffer with 5 % ACN and (see Table 12 in Appendix), while the second protein standard solution (Mix II) contained 0.000427 moles of each protein (see Table 13 in Appendix) dissolved in 50 mM tABC buffer with 5 % ACN.

5.3.3 Cell lysate sample

The cell lysate sample was received from Ph.D. student Tore Vehus. Details for the preparation of lysate samples can be found in section 2.2.5 in Vehus' master thesis [96], and recently submitted manuscript by Hustoft et al. [71].

5.3.4 Reduction and alkylation

A solution of 5 µg/mL DTT was prepared by dissolving 1 mg of DTT in 1 mL H_2O in an Eppendorf vial. The 1 mg/mL DTT solution was diluted 200 times (5 µL of the 1 mg/mL solution and 995 µL H_2O) in a new Eppendorf vial (5 µg/mL DTT). A measured amount of 20 µL of the DTT solution was added to each protein-solution of 5 µg/mL before placed in oven at 56 °C for 45 min. The vials were subsequently cooled to room temperature (25 °C).

A weighed amount of 5 mg IAM was dissolved in 1 mL H_2O (5 mg/mL IAM), and subsequently diluted 200 times (5 µL of the 5 mg/mL IAM solution and 995 µL H_2O), in an Eppendorf vial (25 µg/mL IAM). An aliquot of 10 µL of this alkylation solution was added to each protein sample before placed in the dark for 20 min. The alkylated proteins were then placed in a freezer (-18 °C) until use.

5.3.5 Conventional in-solution digestion

Trypsin, Lys-C or the T/L mixture was added to a protein or mixture at an E+S of 1+25 (w/w). 50 mM tris-HCl (pH 8.0) was used as digestion buffer. The solution was mixed before placed in the thermoshaker from Grant Instruments Ltd. (Cambridge, UK) at 37 °C over night. The digestion was terminated the following day by adding TFA to a final concentration of 0.5-1.0 % (v/v). The sample was now ready for analysis.

5.3.6 On-line OTER digestion

The protein sample, added 50 mM tABC and 5 % ACN, was injected into the OTER (see section 5.6.1 and 5.6.2). The digestion time was 0.5-1 h at 37 °C unless otherwise stated. After digestion, the peptides were loaded into the PS-DVB SPE column, and separated in the PS-DVB PLOT column before MS-detection.

5.4 SEM procedure

A sample of the column/reactor (about 1 cm) was cut off by the use of a capillary cutter, placed on a carbon tape, and image was taken by a FEI Quanta 200 FEG-ESEM (FEI, Hillsboro, OR, USA). The low vacuum mode was initiated while taking the SEM images and a large field detector (LFD) was used to acquire a good image. The parameters for magnitude, working distance, spot size and voltage were altered in order to obtain the best resolution for each sample.

5.5 Microscopes

Motic ST 30C (Motic, Hong Kong, China) microscope was used to study the 50 μm ID SPE columns along the side of the column after polymerization. The polymer appeared black inside the capillary after being dried with N_2, and therefore it was quick and easy to observe if it was partly or completely polymerized.

An Olympus (NO. 501919, Tokyo, Japan) microscope was used to study the end opening of the 20 μm ID HEMA-VDM polymerized capillary and the 10 μm ID PS-DVB PLOT columns after polymerization. The Olympus microscope could magnify the opening of the capillary and whether there was a polymer layer on the capillary wall or not. The columns were cut until there was an even layer of polymer on the inside. This was a faster way to find out where and if the column was polymerized, because taking a SEM image is far more time consuming.

5.6 The LC-MS-system

The OTER, SPE and the PLOT columns were integrated in the manually operated chromatographic system developed by Hanne K. Hustoft, before being transferred to the automatically operating chromatographic system set up by Ph.D. student Tore Vehus. The OTER, SPE and PLOT columns were integrated in the same order as for the manual and the automated system, see Figure 23. The OTER was connected to valve 1 (V1) as shown in Figure 26.

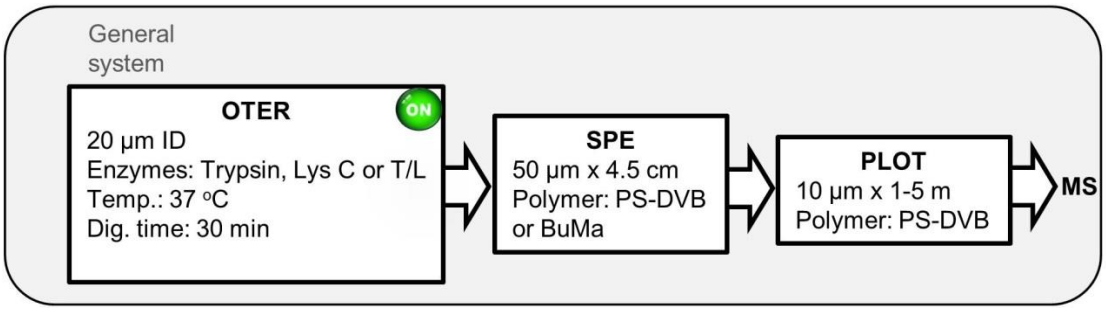

Figure 23: The position of the OTER, SPE and PLOT columns in both the manual and automated system. Digestion temperature (Temp.) and time (Dig. time) was 37 °C and 30 min unless otherwise stated.

5.6.1 Manual LC-MS-system

The manually operated LC-MS-system was developed by Hanne K. Hustoft (Figure 24). The system has been published in Scientific Reports (Nature, 2013) by Hustoft et al. [27], and used for preliminary work in a manuscript submitted by Hustoft et al. to PLoS ONE.

The manual LC-MS-system consisted of three pumps (P). P1 (Agilent 1100 isocratic, Agilent, Sao Paulo, CA) was used for the introduction of the sample into the OTER with a 0.5 µL/min flow rate for 1 min. A second pump, P2 (Agilent 1200 with degasser (G1379A series)), was used for trapping of the peptides from the OTER into the PS-DVB SPE column. The trapping time into the SPE column was 4 min at a flow rate of 0.5 µL/min. Gradient elution of the peptides from the SPE column and into the PS-DVB PLOT column was performed with gradient pump P3 (Agilent 1100 series with a degasser (G1379 series)). The flow rate was set to 2 µL/min, but a splitting of the flow rate of 1/50 at valve 2 (V2) resulted in a flow rate of 40 nL/min through the PLOT column. By varying the length of 10 µm ID fused silica until the measure flow rate was 40 nL/min was used to obtain this split ratio.

In total, three valves were used in the LC-MS-system; one 10-port (V1) and two 6-port valves (V2 and valve 3 (V3)). All valves were purchased from Valco Instruments Co. Inc., Houston, TX. The 10-port valve was placed inside a column oven (Mistral, Spark, The Netherlands) at 37 °C.

A PicoClear™ union connected the PLOT column to a PicoTip™ nanospray tip with a 5 µm ID. Both were purchased from New Objective Woburn, MA, USA.

P1 and P2 used the same mobile phase reservoirs. Mobile phase A consisted of 50 mM NH_4OAc, pH 8.75, and mobile phase B consisted of ACN/NH_4OAc pH 8.75 (90/10, v/v). For trapping of the peptides into the SPE column, 4 % B was used. For P3 the mobile phase reservoir A contained H_2O/FA (100/0.1, v/v), and mobile phase reservoir B contained ACN/H_2O/FA (90/10/0.1, v/v/v). For elution, and separation of the peptides a gradient from 5-40 % B for 5 min was used, and then 40 % B from 5 to 8 min. After each injection, the OTER, SPE and PLOT columns were conditioned with at least 10 column volumes before injecting the next sample.

Two instruments were used for MS detection. MS 1 was a LTQ XL Orbitrap from Thermo Fisher Scientific (Waltham, MA, USA), and it was operated in positive ionization mode with CID fragmentation. The mass range was *m/z* 200-2000, and an ES voltage of 1.3 kV was applied to the ESI needle. MS 2 was a Q Exactive Orbitrap from Thermo Fischer Scientific. It operated in positive ionization mode with higher-energy collision dissociation (HCD) fragmentation, mass range of *m/z* 200-2000, and a 1.3 kV ES voltage on the ESI needle. For both instruments, the data were controlled by Xcalibur software from Thermo Fisher Scientific. For other parameters see automated LC-MS-system in section 5.6.2.

The manual LC-MS-system is also presented as an animation under the following URL http://prezi.com/zxp2ioe_ecp2/lc-system/. The animation has also been used in the publication by Hustoft et al. [27].

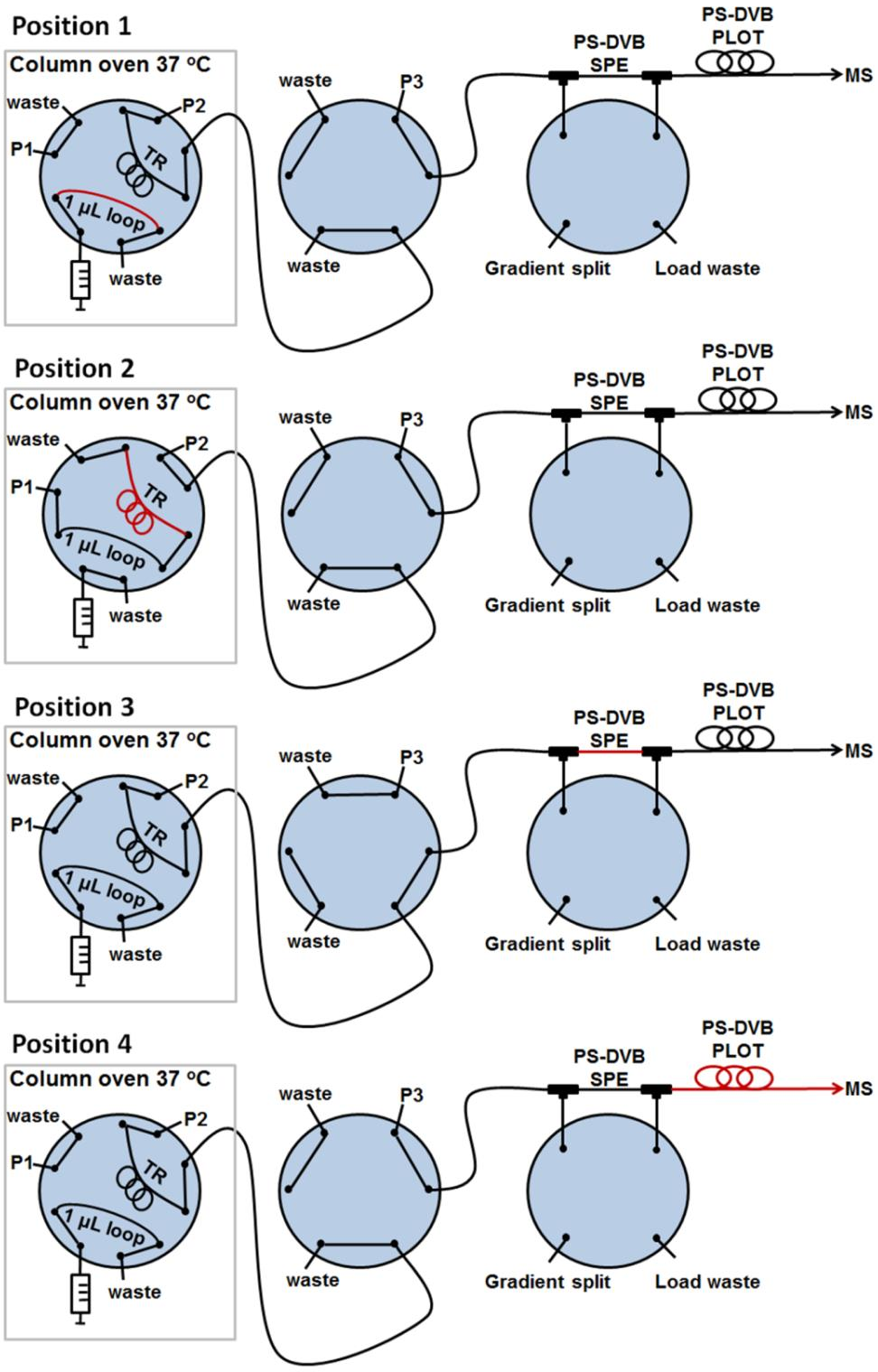

Figure 24: Representation of the manual LC-MS-system also used in a published paper by Hustoft et al. [27] and for preliminary work to the submitted manuscript by Hustoft et al. [71]. Red indicates where the sample is in each position. Flow rate on P1 and P2 was 0.5 µL/min. P3 was set to 2 µL/min, and a 1/50 split of the flow rate means that the pump delivered 40 nL/min in the PLOT column.

5.6.2 Automated LC-MS-system

The automated LC-MS-system was set up by Tore Vehus, and the SPE-PLOT part of the system has been published by Rogeberg et al. [28,41].

The setup in Figure 23 was incorporated in an Easy 1000 nLC system (from Thermo Fisher Scientific (Bremen, Germany)), with an integrated autosampler, injection- and gradient pump, Figure 25. Mobile phase A, of the gradient pump, consisted of H_2O/ACN/FA (96/4/0.1, v/v/v), while mobile phase reservoir B contained ACN /FA (100/0.1, v/v).

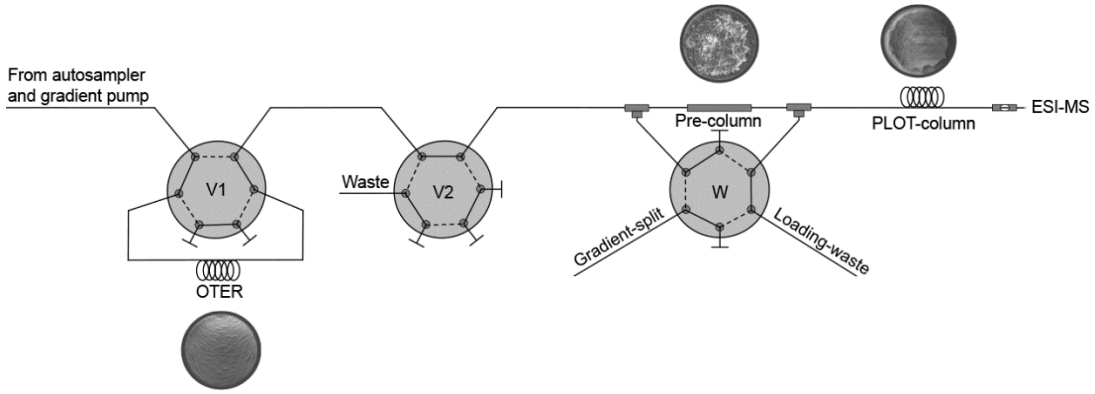

Figure 25: Schematic presentation of the automated LC-MS-system. For detailed positions, see animation for submitted paper by Hustoft et al. [71].

The following details of the workflow in the automated system are also shown in an animation (https://vimeo.com/91700850), and Figure 26 can be helpful for visualization of the process. The animation has also been used in the manuscript by Hustoft et al. [71].

The enzymatic reactor was incorporated into the Easy nLC pump system by utilizing two automatic switching valves. One was a Rheodyne ® 6-port valve (V2, Figure 26, IDEX Health & Science LLC, Rhonert Park, CA, USA) and one was a 10-port valve (V1, Figure 26, VICI, Valco). The automatic switching valves were connected to the MS contact closure outputs and were programmed and controlled by the MS instrument software.

The autosampler in the Easy nLC pump system was programmed to load 5 µL into the injection loop. About 1.2 µL of this sample was loaded into the OTER (ranging from 1–4 m). Four OTERs were connected by PicoClear™ unions in order to achieve an OTER length of 4

m. The sample was delivered to the OTER at a flow rate of 0.5 µL/min (V1 connecting valve positions 1-2 and V2 connecting 1-6, Figure 26).

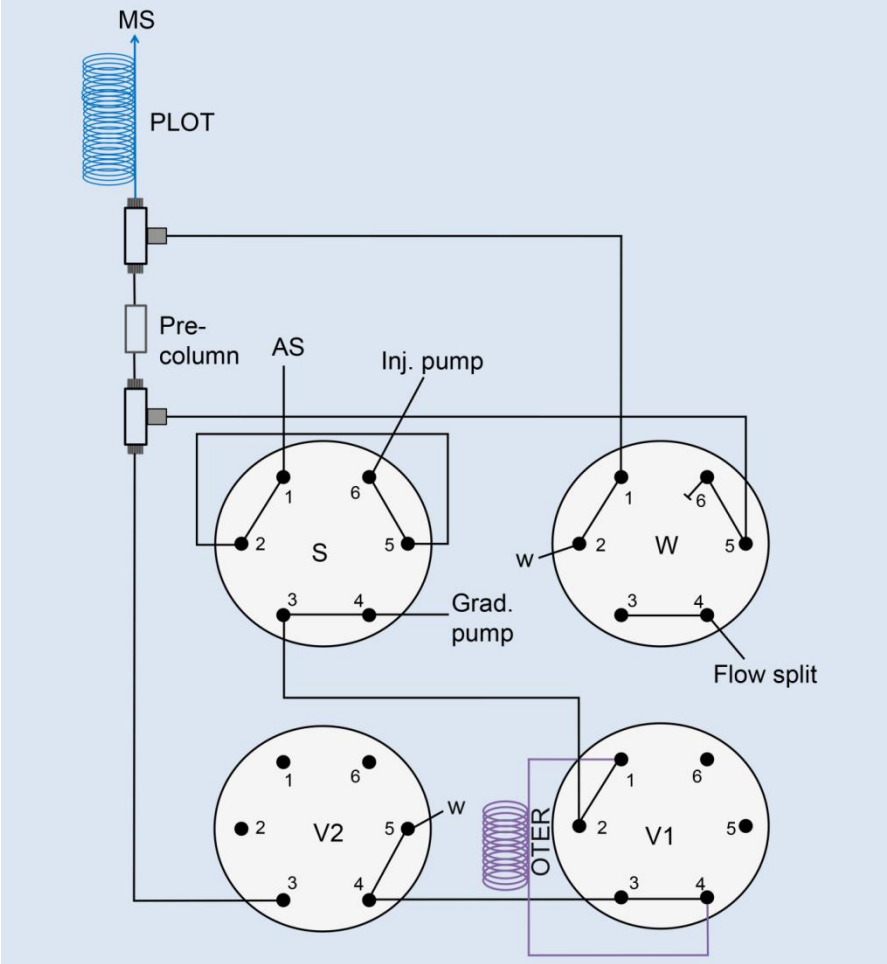

Figure 26: An illustration of the automated LC-MS-system with labelled positions on the valves. The image is taken from the animation and made to illustrate the workflow of the LC-MS-system (https://vimeo.com/91700850 from submitted manuscript by Hustoft et al. [71]).

The OTER was kept in a Mistral column oven set to 37 °C unless otherwise stated. After loading the sample into the OTER, V1 was switched (from 1-2 to 1-6) and the tubing were flushed with 100 % of mobile phase A for 5 min. Meanwhile, the autosampler loop was flushed with 4 % ACN in 50 mM NH_4OAc. After these 5 min, V2 was switched (from 1-2 to 1-6) and the SPE (BuMa 50 µm ID x 4.5 cm) and PLOT (PS-DVB 10 µm ID x 500 cm) columns were equilibrated for 55 min with 40 nL/min.

To elute the peptides off the OTER and into the SPE column, 2 µL of 4 % ACN in 50 mM NH_4OAc was used with valve W (1-2) for 4 min at 0.5 µL/min. The flow rate over the SPE and PLOT column was set to 40 nL/min by switching valve W (1-6) before separation of the peptides in the PS-DVB PLOT column proceeded. By varying the length of 10 µm ID fused silica capillary until the measured flow rate was 40 nL/min was used for obtaining this split ratio.

When performing analysis of in-solution digested protein standard solutions V1 connected 1-6, while V2 connected 1-2. The injection volume of the in-solution digested protein standard solution was 1 µL, and was loaded into the SPE column for 4 min at a flow rate of 0.5 µL/min before separation in the PS-DVB PLOT column proceeded.

For analysis of the protein standard solutions, the peptides eluted from the OTER to the BuMa monolithic SPE column and separation of the peptides used a gradient from 5-40 % B for 30 min. After 30 min, a linear gradient up to 95 % B within 10 min was performed, before the gradient was held at 95 % B for 10 min.

For analysis of the cell lysate sample, a gradient from 5-40 % B for 250 min followed by a linear gradient up to 95 % B in 10 min before held at constant 95 % B for 20 min was used.

The SPE and PLOT columns were conditioned with minimum 10 column volumes before a new analysis was initiated. The OTER was conditioned with minimum 10 column volumes of 50 mM NH_4OAc, pH 8.75 before a new sample was injected.

A Q Exactive Orbitrap mass analyzer from Thermo Fischer Scientific was used for peptide detection. It was operated in positive ionization mode with HCD fragmentation, mass range of *m/z* 200-2000, capillary temperature of 250 $^{\circ}$C and a 1.3 kV ES voltage on the ESI needle. The ESI needle was 10 µm ID to 5±1 µm (PicoTip®, New Objective, Woburn, MA, USA). For full MS, the resolution was set to 75,000 with an automatic gain control (AGC) value of 1e6, and maximum fill time of 120 ms. The MS/MS resolution was set to 35,000 with AGC target value of 1e5 and maximum fill time of 120 ms. The normalized collision energy (NCE) was set to 25. Isolation width of *m/z* 2.0 was chosen and only ions with charge +2 to +6 were selected for fragmentation. The dynamic exclusion was set to 30 seconds. The data were controlled and analyzed by Xcalibur software (v2.2, Thermo Fisher Scientific).

5.7 Search parameters

The search parameters were as described in the manuscript by Hustoft et al. [71].

Proteome Discover™ (version 1.4, Thermo Fisher Scientific) was used to calculate the SQ % for each sample injection. A Sequest algorithm was used to identify the peptides and the corresponding proteins from the MS/MS fragment spectra for the single protein standards and mixtures of protein standards digested in the OTERs. The data from the cell lysate samples were cross-validated with both Mascot and Sequest.

Maximum two missed cleavages were allowed, 10 ppm precursor mass tolerance and a fragment mass tolerance of 0.8 Da were used. In addition, a false discovery rate of 1 % was used, i.e. the sets of data used to calculate the SQ % are 99 % confident. The enzyme specificity was either trypsin or Lys-C, and when combination of these enzymes was used for digestion, the data were searched with both enzymes and merged.

Restrictions were set to the SQ % results. A medium peptide confidence level was used for the protein standards, while high peptide confidence level was used for the cell lysate sample.

6 Results and Discussion

During this study, optimizing and development of OTERs were investigated. BuMa and PS-DVB monolithic SPE columns and PS-DVB PLOT columns that were needed in the test system were reproduced as described in previous published papers [26,94,95]. A schematic illustration of the chromatographic system used in each data set is presented so that the reader will have a better understanding while reading the results. Details from the schematic illustrations can be found in the experimental section. An overview of the investigations carried out in the present thesis is presented in Figure 27.

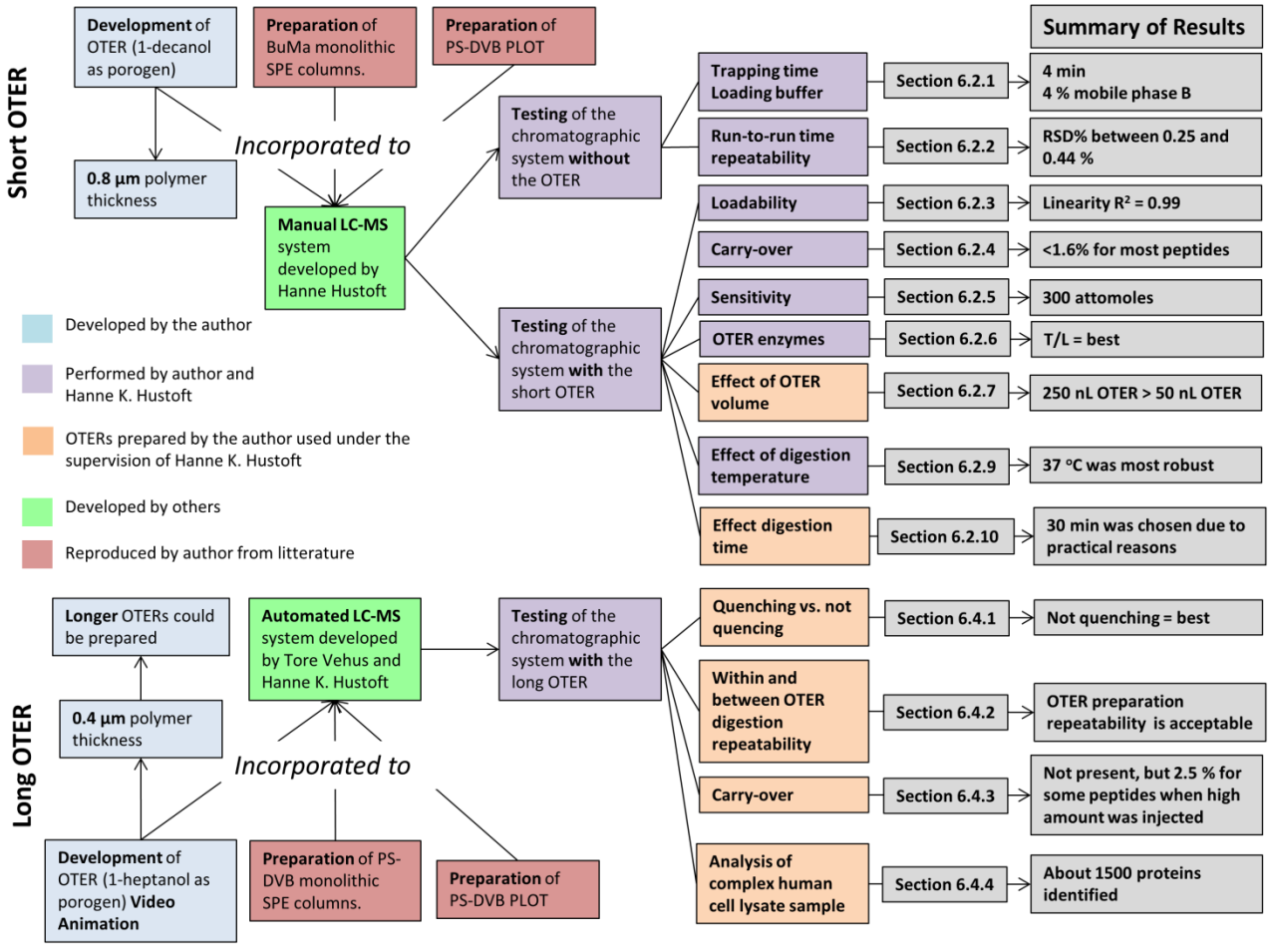

Figure 27: Overview of the investigations carried out in the present thesis. The color codes of the boxes are explained in the figure.

6.1 Preparation of columns

In the following section the observations and results from the preparation of monolithic SPE columns, PLOT columns and OTERs are discussed.

6.1.1 Monolithic SPE columns

BuMa and PS-DVB monolithic SPE columns were prepared for trapping of peptides as described in the experimental section. From Figure 28, the PS-DVB (left) and BuMa (right) polymer is clearly attached to the inside of the capillary. During SEM imaging, it was observed that the polymerization was homogeneous and contains both macro- (> 50 nm) and mesopores (2-50 nm). Micropores (< 2 nm) were also present; however, they were too small to be observed. The columns could handle a back-pressure up to 400 bar without any bleeding of the polymer. Hence, enough vinyl groups have been silanized into the capillary so that the polymer is well anchored to the inside of the wall.

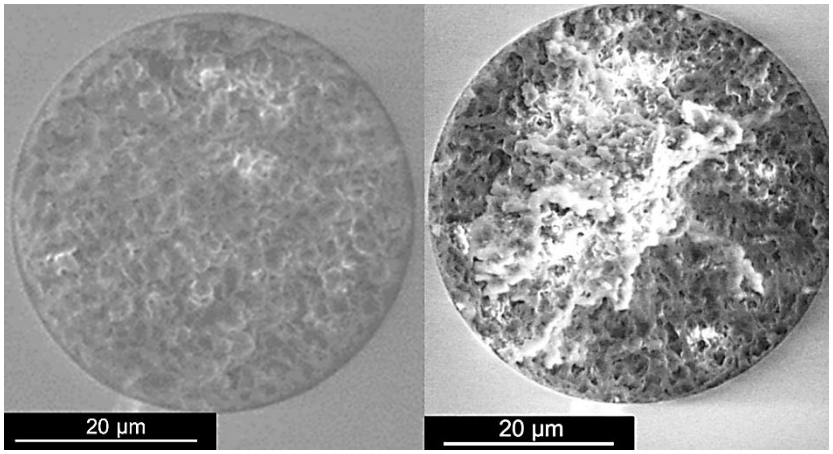

Figure 28: SEM image of 50 µm ID PS-DVB monolith (left) and 50 µm ID BuMa monolith (right). The image was taken in low vacuum while using a LFD, and a high voltage of 12 kV.

The PS-DVB monolith was used in the manual test system for trapping of peptides. When the project reached the point of automation, the BuMa column was already incorporated into the automated system showing good results. Hence, the BuMa column continued as SPE column for the automated experiments.

The BuMa monolithic SPE column was based on a recipe by Geiser et al. [95]. The column-to-column reproducibility of the BuMa monolithic column was not tested in the present study. However, Geiser et al. evaluated it to be between 0.8 and 1.4 % RSD for thermal polymerization [95]. An even better reproducibility (down to 0.5 % RSD) was achieved when they used photochemical initiation. As instrumentation for photochemical initiation was not available in our lab, thermal initiation was carried out. A copolymerization between BuMa and EDMA (Figure 29) creates the polymer network for the BuMa monolith inside the column (Figure 28, right).

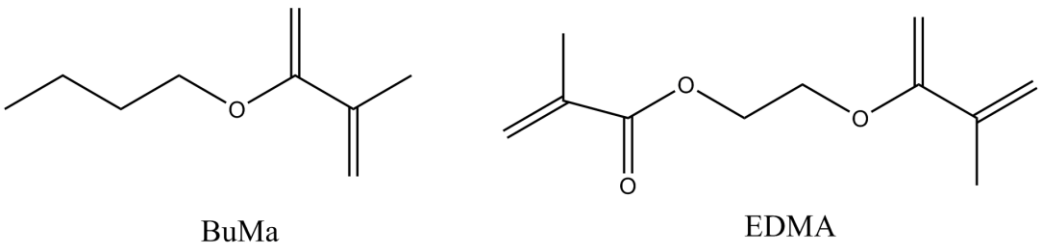

Figure 29: Chemical structures of BuMa and EDMA, which copolymerize into the BuMa monolithic SPE column.

The PS-DVB column was based on the recipe by Lv et al. [94]. However, 4-methylstyrene was replaced with styrene, and vinylbenzene chloride was removed from the original recipe. The monomers, styrene and DVB (Figure 30) copolymerized, forming a network into the polymer on the inside of the PS-DVB monolithic column (Figure 28, left).

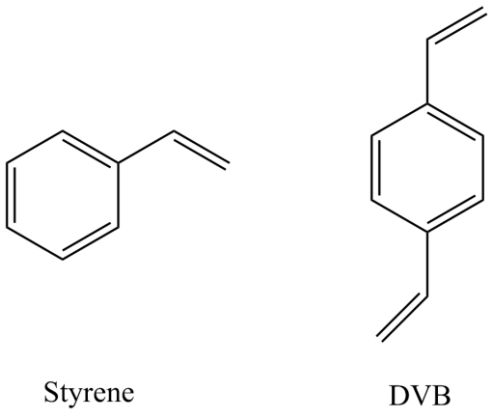

Figure 30: Styrene and DVB that copolymerize into the polymer on the inside of the capillary of the PS-DVB monolithic column.

The preparation of both PS-DVB and BuMa monolithic columns were carried out without any difficulties. N (measured by master student Rena Record) of the BuMa monolithic columns varied from batch to batch (data not shown), ranging from 8,000-20,000 plates per meter. Hence, it is difficult to obtain repeatable efficiency of these monolithic columns.

However, as the main purpose of the PS-DVB and BuMa SPE columns were to trap the peptides originating from the proteins digested in the OTER; the low efficiency columns also served this purpose well (see section 6.4.1).

6.1.2 PLOT columns

In 2010, Rogeberg et al. reported that when polymerizing PS-DVB PLOT columns (10 µm ID) that was used for peptide separation about 5 to 15 cm of each end was either not completely filled, or not filled at all with polymerization solution [41]. The part that was not polymerized was removed by a capillary cutter to ensure that the whole column length contained polymer. This was also done in this study. The film thickness inside the PS-DVB PLOT column was 0.75 µm (Figure 31), and was obtained using the optimized conditions found by Rogeberg et al. [41]. The optimized polymer thickness allowed production of longer PS-DVB PLOT columns and lower back-pressure. PLOT columns ranging from 1-5.5 m were prepared in this project. Longer PLOT columns up to 10 m are possible, but were not attempted to be made for this study.

PLOT columns were found to be easy to reproduce with the optimized standard operation procedure by Rogeberg et al., who also reported that the column-to-column reproducibility was between 2 and 3 % RSD [41].

The polymer on the inside of the 10 µm ID PLOT columns prepared for this study was not possible to study with the Motic microscope, which was used to study the column from the side. Therefore, the Olympus microscope was used, because it could study the capillary from the end (Figure 32). Right after polymerization the column was studied with this microscope so that the unpolymerized part could be cut off.

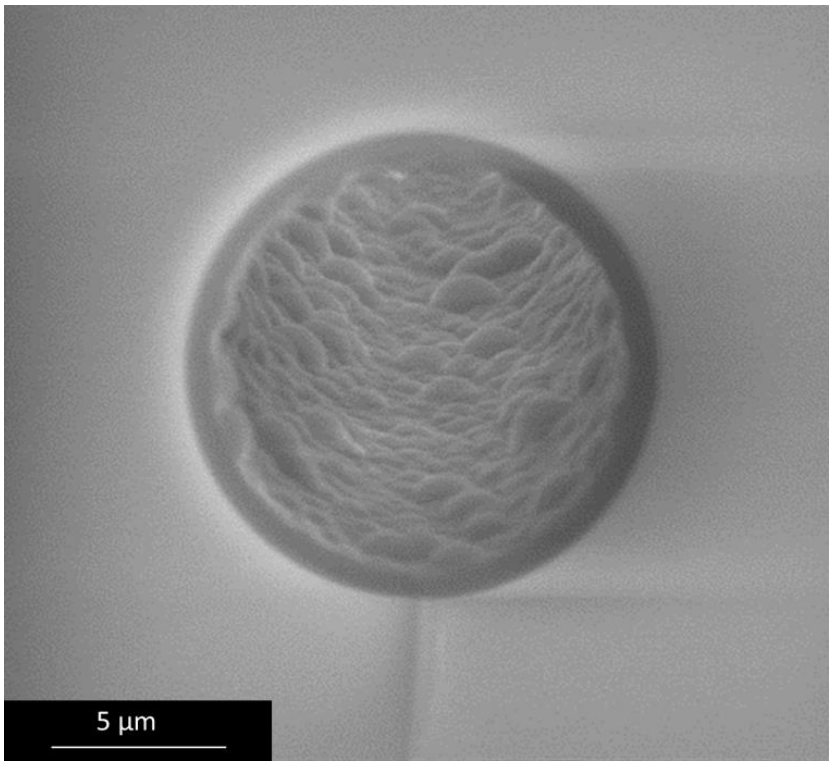

Figure 31: SEM image of 10 µm ID PS-DVB PLOT column with 0.75 µm film thickness. The configuration of the SEM in order to be able to take the images was low vacuum, with LFD, and high voltage 12 kV.

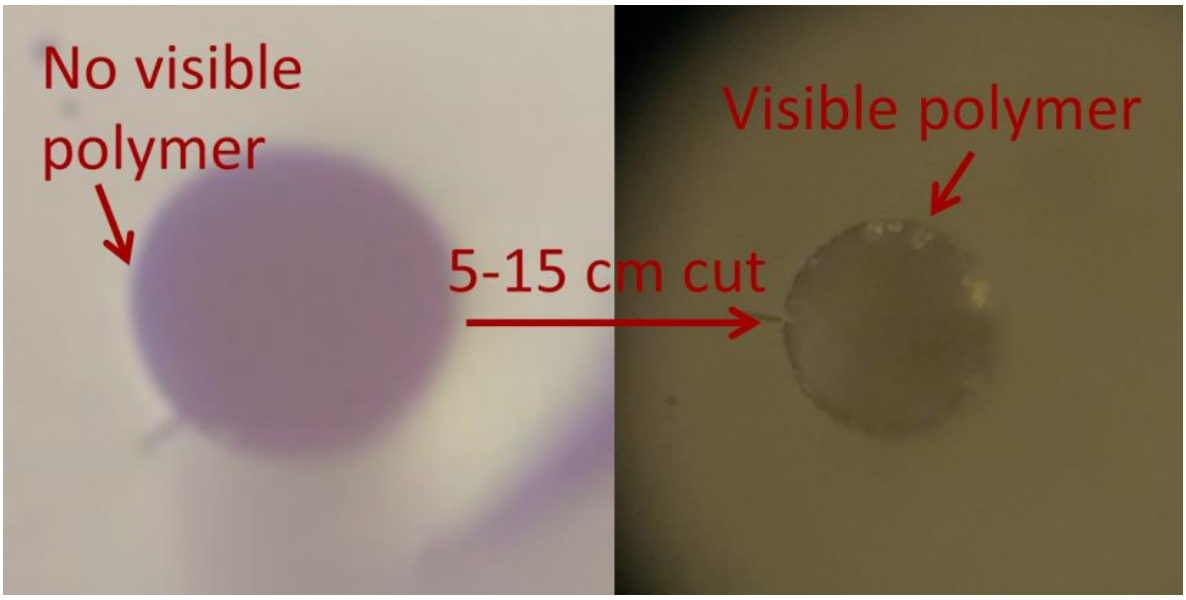

Figure 32: Microscope image of the opening of a PS-DVB PLOT column after polymerization. The part that was not polymerized was between 5 and 15 cm (left). The polymer layer is clearly visible after cutting (right).

6.1.3 OTERs produced with 1-decanol as porogen

The main focus of this study was optimizing and development of OTERs based on a HEMA-VDM polymer. These were prepared in order to perform sample preparation on-line by digesting proteins into peptides. The author has prepared all OTERs and contributed to the testing and evaluation under supervision of Hanne K. Hustoft.

OTERs vs. monolithic IMER

Monolithic IMERs provide a high surface area for enzyme immobilization. However they have rather high back-pressures compared to the open format provided by the OTERs. In the present study, OTER was chosen over the monolithic IMER. IMERs, being monolithic, are more difficult to reproduce, as can be confirmed by the large difference in N when producing the BuMa monolithic columns (see section 6.1.1). In order to compensate for the large surface area that the monolithic columns possess, long OTERs must be produced to immobilize sufficient amount of enzyme. Abele et al. mention that an OT capillary is that it is easy to flush the enzyme solution through, and they possess high surface area if the polymer layer is porous [69].

OTER characterization by SEM imaging was performed in this study, but it is not sufficient to conclude whether the polymer layer was porous or not. A capillary ID of 20 μm was used for the development of OTER in this study. However, other capillary IDs can also be used for the preparation of OTERs.

Post-polymerization observations

After the polymerization step at 65 $^{\circ}$C for 5 h and 80 $^{\circ}$C for 5 h, the OTER was dried with N_2 and studied with the Motic microscope (see section 5.5). It was observed that 5 to 10 cm of the capillary was not completely wall polymerized or not polymerized at all (Figure 33). This was also observed for the PLOT columns (see Section 6.1.2). The lack of polymer layer at the capillary ends could be a result from drying of the polymerization solution from each side, or maybe that the septum did not completely seal the capillary before it was placed in the oven. From previous observations and experience, when the polymerization solution is dried, it clogs the capillary and no liquid or N_2 will go through. Hence, these 5-15 cm were cut off before proceeding with the washing step and immobilization step.

Another theory for the lack of polymer at the ends could be due to poor silanization at the end of the capillary. This would lead to fewer anchoring sites for the polymer. This is more unlikely, because if this was the case, then there should have been fewer anchoring sites along the whole column and it should have been partly polymerized throughout the whole column.

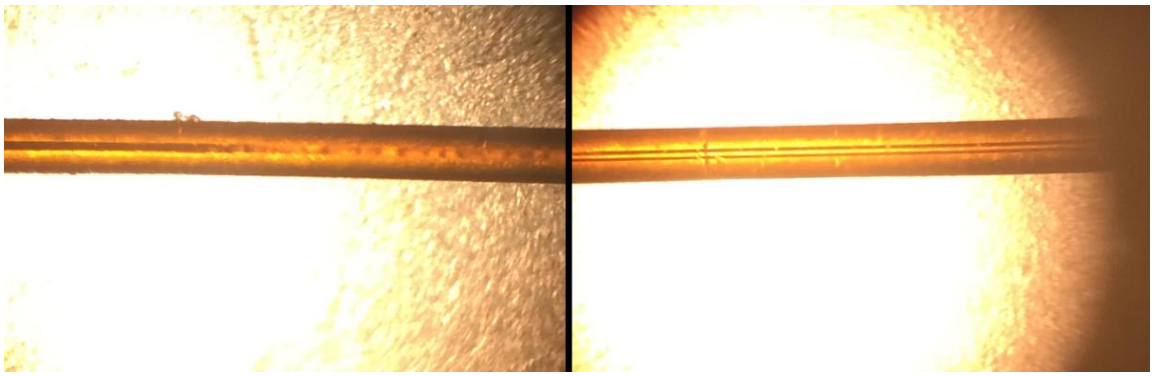

Figure 33: The end of the OTER after polymerization. Partly polymerized (left) and not polymerized at all (right). The polymer appears black in the microscope when dried with N_2.

The HEMA-VDM polymer layer

The polymer is formed by a copolymerization of the two monomers HEMA and VDM, which contain vinyl functional groups (see Figure 34). HEMA is hydrophilic, and is used to increase the hydrophilicity of the column so that there are little hydrophobic interactions between the polymer and the peptides, or undigested proteins. Since the polymer layer was hydrophilic, the mobile phase that was needed to elute the peptides from the OTER and into the SPE column could consist of little ACN. As will be described later, this is important in order to get sufficient trapping of the peptides into the SPE column and to avoid breakthrough.

After polymerization the monomers that did not copolymerize were flushed out by N_2. Whether or not N_2 was sufficient to remove the remaining monomers, was discussed, but since immobilization was done afterwards, any residual monomers could be removed during this process.

VDM is used for the attachment of the enzyme onto the polymer through azlactone groups of VDM and primary amino groups of the enzyme.

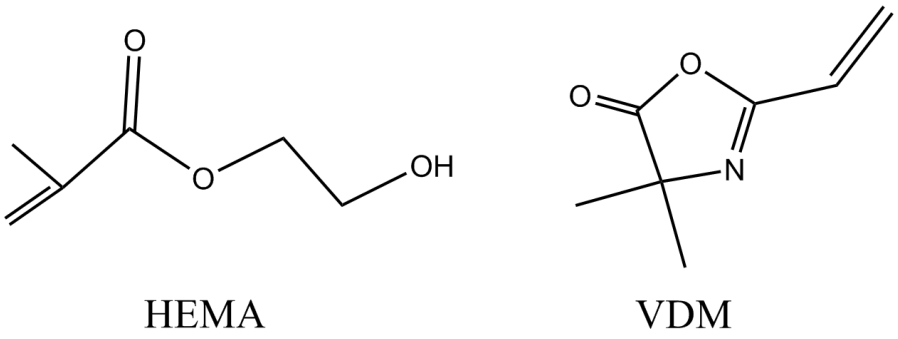

Figure 34: Structures of the monomers HEMA and VDM that copolymerize into a polymer layer inside the 20 µm ID capillaries.

In order to immobilize more enzymes onto the polymer inside the column, and hence increase enzymatic activity inside the OTER, the polymerization step was examined. Huang et al. [97] tested different monomer concentrations and discovered that if the concentration of monomers was high, i.e. above 20 %, a higher amount of unbonded polymer was present. By using this information, we altered the polymerization solution composition in hope of being able to achieve a thicker polymer layer on the inside of the column so that more enzymes would be immobilized (see section 6.3.2).

Immobilization with enzymes

The immobilization reaction of trypsin, Lys-C and T/L onto the HEMA-VDM polymer is shown in Figure 21 in the Experimental section 5.2.4. For the attachment of the enzyme onto the polymer, the VDM covalently attaches the enzyme to the stationary phase through azlactone groups from the VDM and primary amino groups of the enzyme.

Immobilization of enzyme onto the stationary phases can also be through adsorption [98-100], encapsulation [101,102] and entrapment [103,104], to mention some. The review by Safdar et al. discussed different enzymes that were immobilized onto various stationary phases [92]. The most frequently used enzyme for immobilization that is mentioned in this review was trypsin. Other mentioned enzymes were chymotrypsin, proteinase K and Peptide-N-Glycosidase F (also known as PNGase F). Lys-C can also be immobilized onto a stationary phase [105].

It is said that the enzyme to substrate ratio inside the OTER is large [74]. However, we have not calculated the enzyme to substrate ratio in the OTER during digestion, but digestion of mixture of 10 proteins has shown that it is sufficient.

Choice of ID of OTER

It was thought that proteins would have more difficulties to diffuse from the inside of the OTER towards the enzyme attached to the wall as the ID of the OTER increased (>20 μm ID). Hence, larger ID would result in less peptide generation. OTERs with 20 μm ID were prepared in this study, but 10 μm would give faster diffusion of proteins to the trypsin immobilized on the polymer attached to the wall. However, there was a problem with achieving a polymer layer on the inside of a 10 μm ID capillary using the same polymerization conditions as for the 20 μm ID capillary. In addition to that, OTERs prepared in 10 μm ID would give a higher back-pressure in the system, which means that sample loading into the OTER would demand a lower flow rate. This would be more time consuming.

In the author's opinion, optimizations of polymerization conditions have to be performed for each capillary ID. Considering the time aspect during this study, this was not prioritized.

6.2 Optimizing the manual LC-MS-system

The manual chromatographic system was optimized under the supervision of Hanne K. Hustoft. Results have been published [27] (Figure 69 in Appendix), and used for a submitted manuscript by Hustoft et al. [71]. In addition, the results have also been presented at Kjemi Grand Prix (Figure 67) and during Kromatografisymposiet in Sandefjord (Figure 68).

6.2.1 Optimizing the SPE column trapping time and loading buffer

Adjusting parameters as trapping time and amount of ACN in the loading buffer from the OTER and into the SPE column for peptide trapping are very important in order to achieve the highest amount of peptides into the MS for detection.

In-solution digested Myo was used for the optimization of the chromatographic system. The OTER was exchanged with an empty fused silica capillary since the protein was in-solution digested. The peak areas of four extracted peptides were used to evaluate the performance. For summary of which system, sample and columns that was used for the following optimizations, see Figure 35.

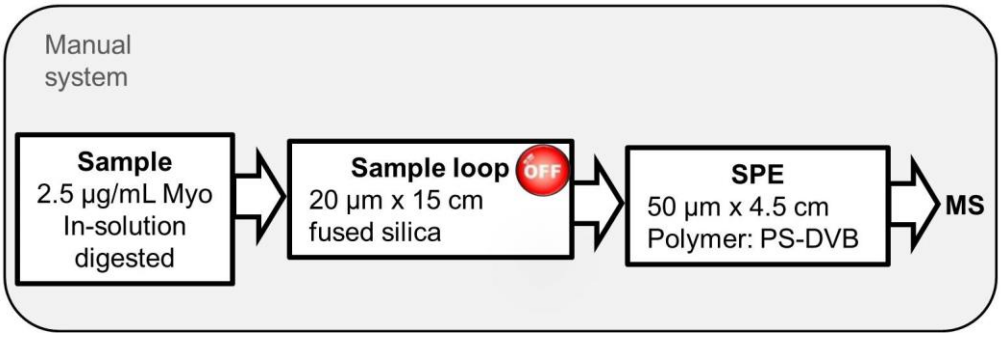

Figure 35: Schematic illustration of the highlights in the chromatographic system that was used for optimizing of trapping time and loading buffer from the fused silica capillary and into the SPE column.

Optimizing the SPE column trapping time

Optimization of the trapping time, i.e. the time needed to transfer the peptides from the fused silica capillary to the 50 µm ID x 4.5 cm PS-DVB SPE column was done without the PLOT column for simplicity. The peptides might not be completely trapped into the SPE column if the trapping time is too short. Breakthrough can occur if the peptides elute off the SPE column to waste during trapping.

This study was carried out by injecting 2.5 µg/mL in-solution digested Myo and an empty 20 µm ID x 15 cm fused silica capillary (6.9 femtomoles) for loading the sample.

When varying the trapping time from 1 to 6 min, 4 min was found to be the trapping time that resulted in highest capture of the peptides (Figure 36, data shown in Table 9 in Appendix). This was done in the manual LC-MS-system with a flow rate of 0.5 µL/min. Therefore, 4 min was used in the later chromatographic systems for trapping of the peptides.

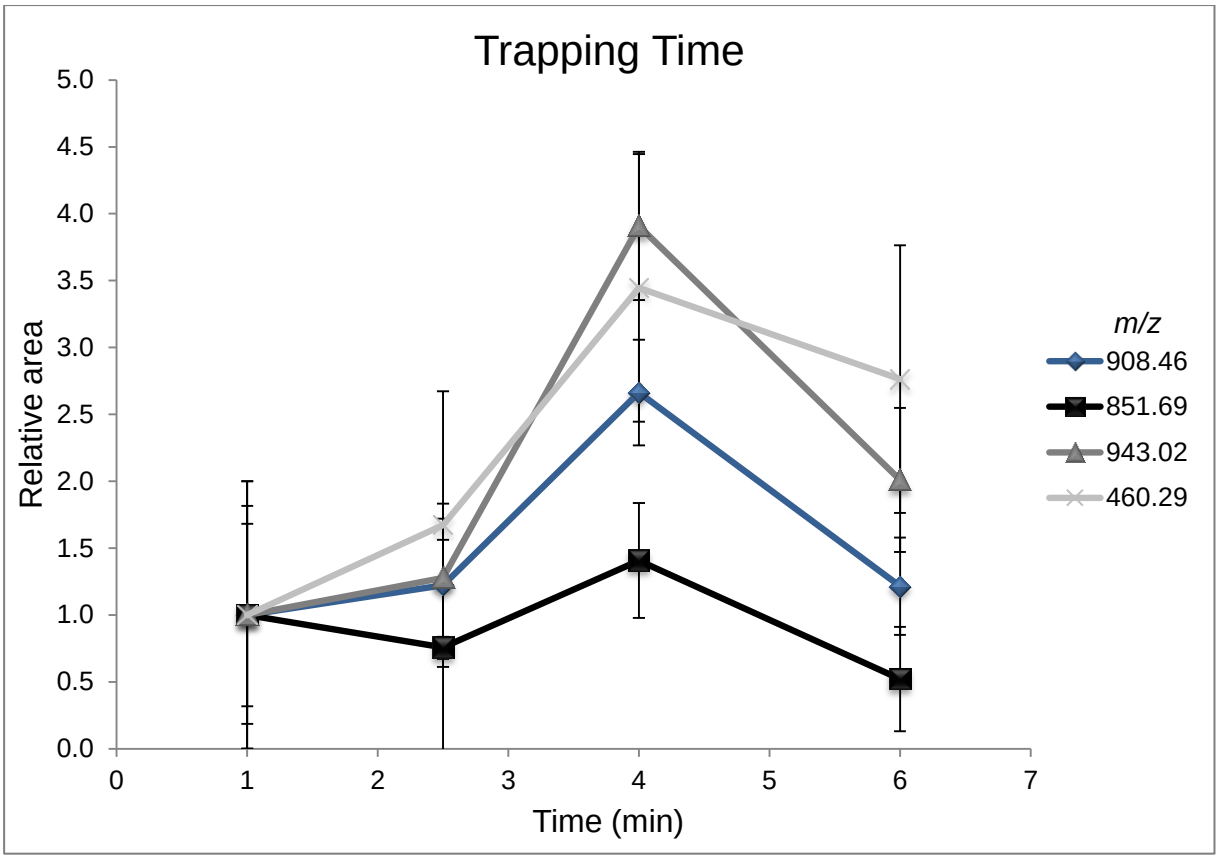

Figure 36: Relative peptide peak area of 2.5 µg/mL in-solution digested Myo. Extracted ion chromatogram (EIC) of peptides with *m/z* 908.46, 851.69, 943.02 and 460.29 from Myo as a function of SPE column trapping time is shown. Replicate injections (rep, n = 3).

Optimizing the SPE column loading buffer composition

The loading buffer used to elute the peptides off the OTER and trapping in the SPE column had to be optimized. The loading buffer consisted of A: 50 mM ammonium acetate (NH_4OAc, pH 8.75) and B: 10 % 50 mM NH_4OAc (pH 8.75)/90 % ACN (v/v).

The peptides may have some hydrophobic interactions with the OTER, even though the polymer layer consists of hydrophilic monomers (see Figure 34). Hence, the use of some organic modifier may be necessary to elute peptides from the OTER.

Optimization of the loading buffer was carried out by injecting 2.5 µg/mL (6.9 femtomoles) in-solution digested Myo, Figure 37 (data shown in Table 10 in Appendix). By adjusting the percent of mobile phase B from 1-10 %, 4% was observed to trap the highest amount of peptides in the manual LC-MS-system. Loading buffer consisting of 4 % of mobile phase B was therefore used for eluting the peptides from the OTER and into the SPE column in later experiments.

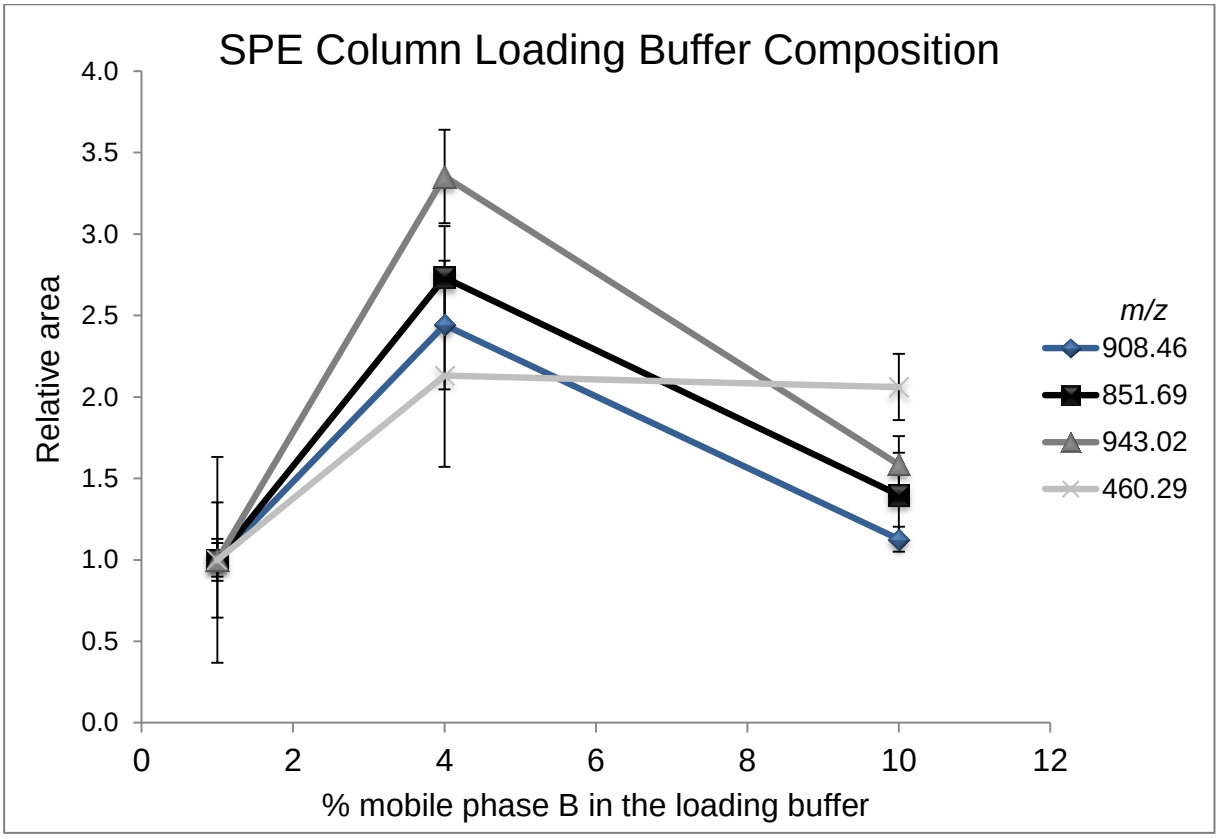

Figure 37: Relative peptide peak area from EIC of extracted peptides with *m/z* 908.46, 851.69, 943.02 and 460.29 from Myo when increasing the % mobile phase B in the loading buffer (n = 3).

6.2.2 Run-to-run retention time repeatability of the manual SPE-PLOT LC-MS-system

Run-to-run retention time repeatability is important to study because this can be used to evaluate to which extent the peptides undergo equal workflow from run to run. This is especially important in targeted MS detection where the MS is programmed to take MS/MS within a specific retention time window.

The repeatability of the SPE-PLOT system was assessed by three replicate injections of 2.5 µg/mL in-solution digested Cyt C (Table 5). The in-solution digested Cyt C (10 femtomoles) peptide sample was loaded into a 20 µm ID x 15 cm empty fused silica capillary before trapped in the 50 µm ID x 4.5 cm PS-DVB SPE column. The peptides were separated with a 10 µm ID x 100 cm PS-DVB PLOT column before MS detection. Figure 38 is a sketch of the highlights for the chromatographic system that was used when the run-to-run retention time repeatability was tested.

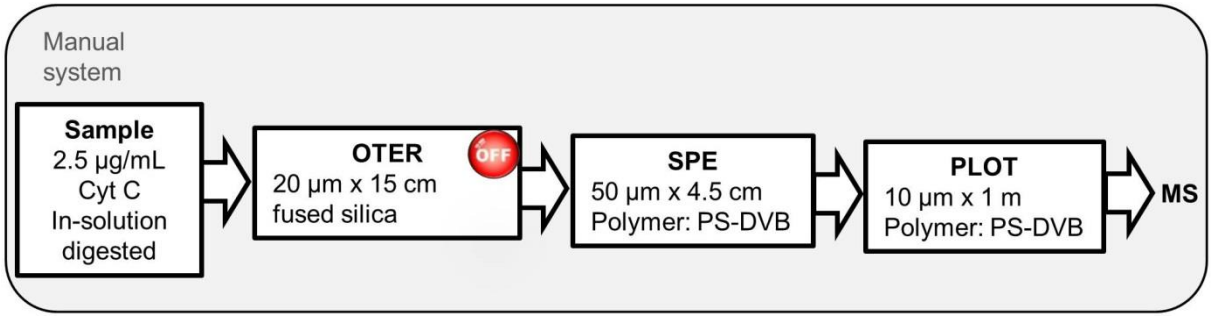

Figure 38: The highlights of the chromatographic system that was used to test the run-to-run repeatability of the manual SPE-PLOT LC-MS-system.

The retention times of three peptides (*m/z* 792.88, 728.83 and 1005.48) were examined, Table 5. The RSD % of the retention time of three extracted peptides from three replicate injections was between 0.25 to 0.44 %. This shows that the manual LC-MS-system provided sufficient repeatable retention times. The RSD of the run-to-run retention time repeatability was almost 3.5 times lower compared to that of Yue et al. [26], who could report a RSD of 1.2 %. The system shows great potential to be used for targeted MS in the future.

Table 5: Retention time (min) of 3 replicate injections of 3 in-solution digested Cyt C peptides (n = 3)

Peptides *m/z*	792.88	728.83	1005.48
Rep 1	13.68	13.90	18.16
Rep 2	13.62	13.80	18.00
Rep 3	13.68	13.87	18.08
Average	13.66	13.86	18.08
St. Dev.	0.03	0.05	0.08
RSD %	0.25	0.37	0.44

6.2.3 Loadability of the OTER-SPE-PLOT manual LC-MS-system

Loadability of the manual system, i.e. how much sample the system can handle, was examined in order to investigate the maximum sample concentration that could be injected until breakthrough was observed. For this examination a tumor marker for small cell lung cancer ProGRP (obtained as described in [93]) was used. The highlights in the chromatographic system that was used for testing of the loadability are shown in Figure 39.

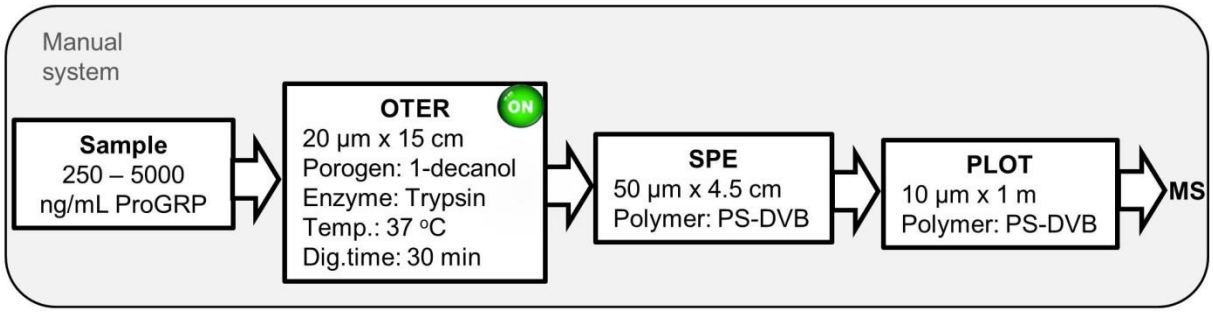

Figure 39: Highlights of the chromatographic system that was used to evaluate the loadability of the OTER-SPE-PLOT manual LC-MS-system.

Loadability was evaluated by analyzing increasing concentration from 250 to 5000 ng/mL of on-line digested ProGRP (0.6-12 femtomoles). Plotting the peak areas of the EIC (Figure 40, above) for the signature peptide NLLGLIEAK (485.8^{2+}) (Figure 40, below, data shown in Table 11 in Appendix) gave a linearity of $R^2 = 0.99$. Hence, the system could, without any

breakthrough, handle an amount of at least 12 femtomoles of ProGRP (5000 ng/mL ProGRP in a 20 µm ID x 15 cm OTER).

Loading a higher amount of sample into the system is not realistic, because concentrations of a protein in real samples will most likely not exceed 12 femtomoles. In addition to that, when preparing protein standards above 5 µg/mL, sedimentation and crystallization of the protein was observed. Spraying high concentrations of undigested proteins into the MS is undesirable. Proteins will stick to the inside of the MS, and a thorough cleaning and calibration of the MS have to be done afterwards.

The absence of breakthrough and linearity of peptides peak area when injecting a high concentration, such as 12 attomoles ProGRP shows that the system is very robust and can tolerate a high amount of sample.

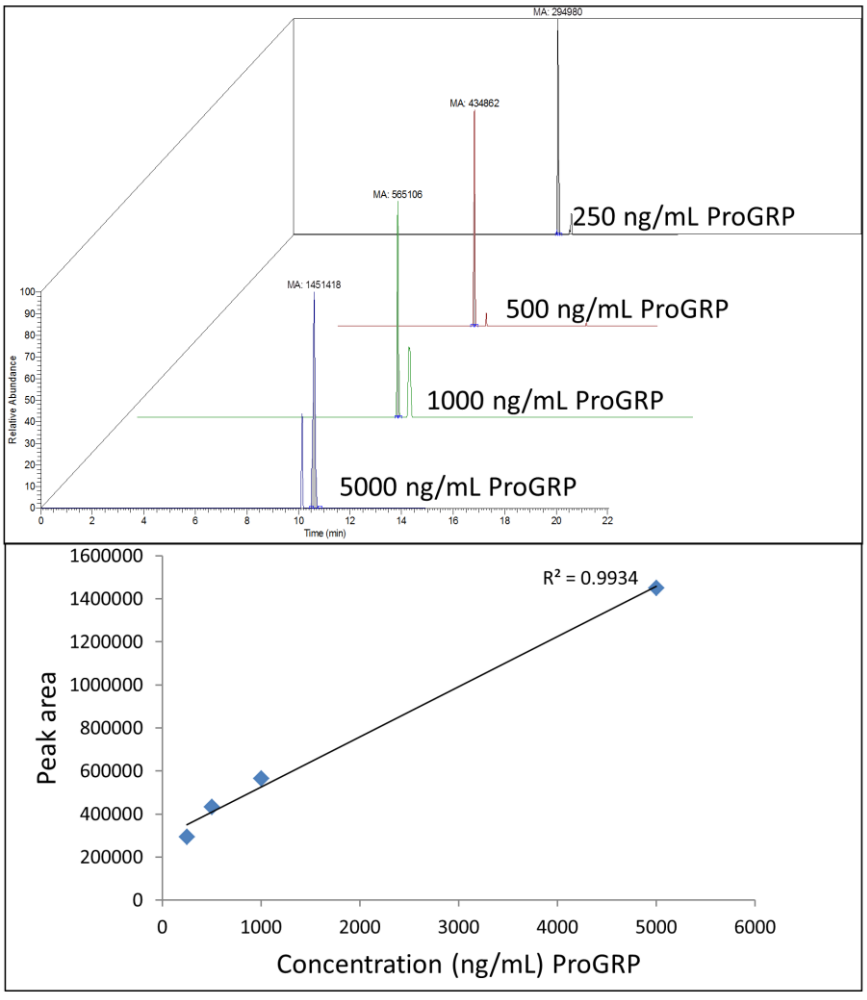

Figure 40: EIC of signature peptide NLLGLIEAK (485.8^{2+}) of increasing concentrations of ProGRP (above). Peak area plotted against concentration (below).

6.2.4 Carry-over

Extent of carry-over, i.e. traces of the previous sample in the system after an injection, is used to control the quality of the system [49]. A false positive could be reported in the following injection if the system contains carry-over. Carry-over can be investigated with a blank injection. The fewer blank injections needed after a sample analysis to ascertain no carry-over, the more time efficient is the sample analysis.

The carry-over of the whole system (Figure 41) was assessed by injecting 40 nL of 100 ng/mL intact ProGRP (=300 attomoles) followed by a blank injection, see Figure 42. This is a very low amount of protein, and was not expected to give high carry-over issues.

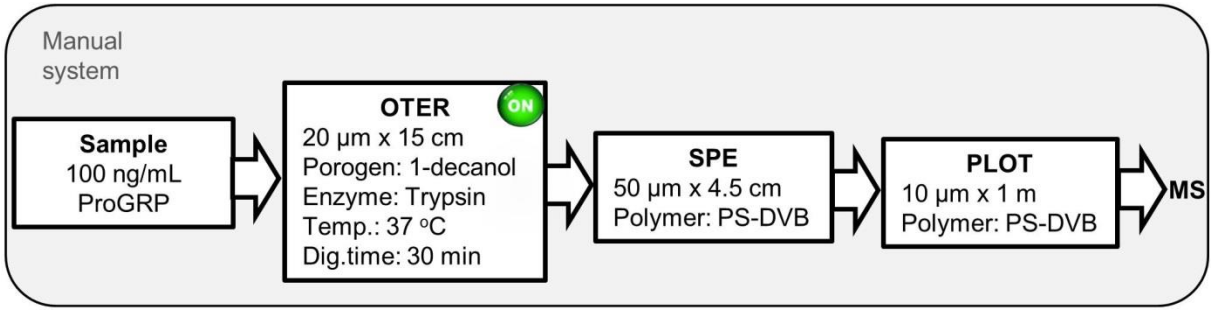

Figure 41: Highlights of the chromatographic system that was used to study the carry-over.

Comparing EIC of the signature peptide NLLGLIEAK (485.8^{2+}) for the on-line digested sample of ProGRP and the following blank injection was performed to evaluate the carry-over in the system. No carry-over was observed with this concentration. Carry-over was monitored during the study and it was found to be less than 1.6 % for most peptides on a general basis, with the exception of 8 % carry-over for one Myo peptide (*m/z* 460.29, HGTVVLTALGGILK), which was observed in one sample analysis.

This means that the samples could be analyzed subsequently without a following blank injection. This reduced the time consumption of the study tremendously. However, carry-over tests were performed throughout the study.

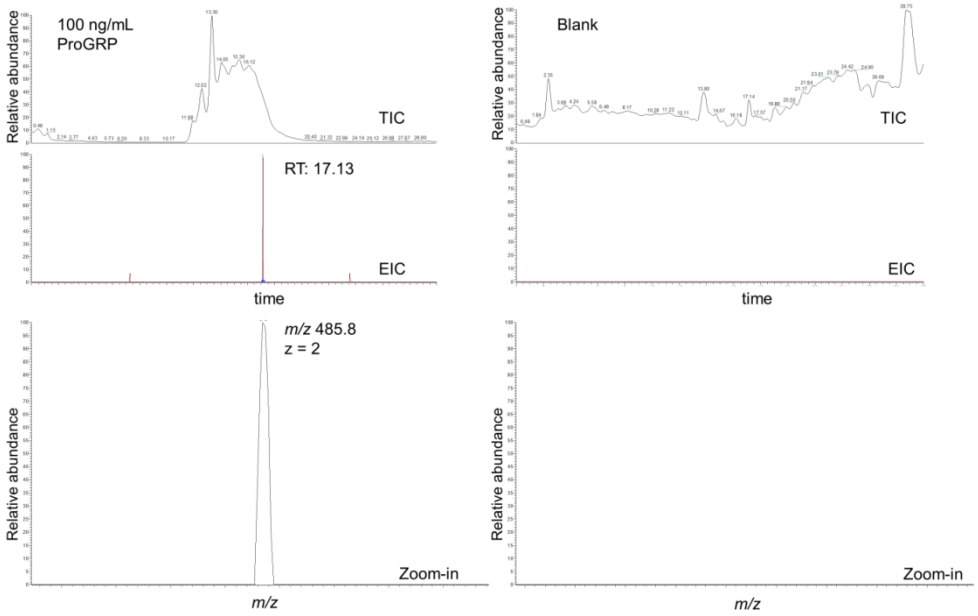

Figure 42: Left: Chromatogram of 100 ng/mL on-line digested ProGRP. Upper: Total ion chromatogram (TIC), middle: EIC of *m/z* 485.8^{2+}, lower: fragmentation spectrum. Right: Chromatogram of blank injection following the 100 ng/mL injection of on-line digested ProGRP. Upper: TIC, middle: EIC of *m/z* 485.8^{2+}, lower: fragmentation spectrum.

6.2.5 Sensitivity of the OTER-SPE-PLOT manual system

The manual system developed by Hanne K. Hustoft [27] (explained in section 5.6.1) could separate and detect low concentrations of ProGRP. An injection of 300 attomoles on-line digested ProGRP (reference limit levels of 58.9 pg/mL = 7.6 picomole [93]) in the OTER-SPE-PLOT system (Figure 43) was compared with an injection of 2500 attomoles in-solution digested ProGRP (5 times estimated limit of detection (LOD) of 1 picomole) as described by Torsetnes et al. [93].

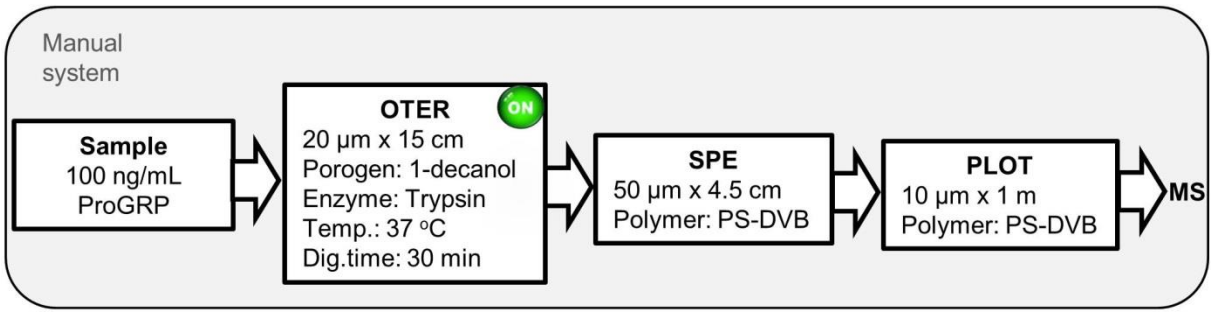

Figure 43: Highlights in the chromatographic system that was used to evaluate the sensitivity of the OTER-SPE-PLOT manual system.

The EIC of the signature peptide NLLGLIEAK (485.8^{2+}) for the two injections are presented in Figure 44. The retention-windows have been adjusted so that they are relative in time. In the present system, injection of 300 attomoles resulted in a peak width ($W_{0.1}$) of 3.6 seconds (at 10 % of peak height). The injection of 2500 attomoles using conventional packed columns, in the study by Torsetnes et al. [93], resulted in $W_{0.1}$ of 30 seconds. Having a well-defined charge and mass fragment spectrum, the signature peptide of ProGRP was clearly digested by the OTER and analyzed by the developed manual system.

In this study, miniaturized columns were used (50 μm x 4.5 cm SPE column and 10 μm x 100 cm analytical column), while conventional columns (1 mm x 10 mm SPE column and 1 mm x 50 mm analytical column) were used in the chromatographic system of Torsetnes et al. [93]. Figure 44 (a) clearly shows the improved sensitivity when using miniaturized columns compared to conventional ones in Figure 44 (b). This can be explained by the fact that the column ID is inversely proportional with signal intensity when using concentration sensitive detectors like ES-MS [106].

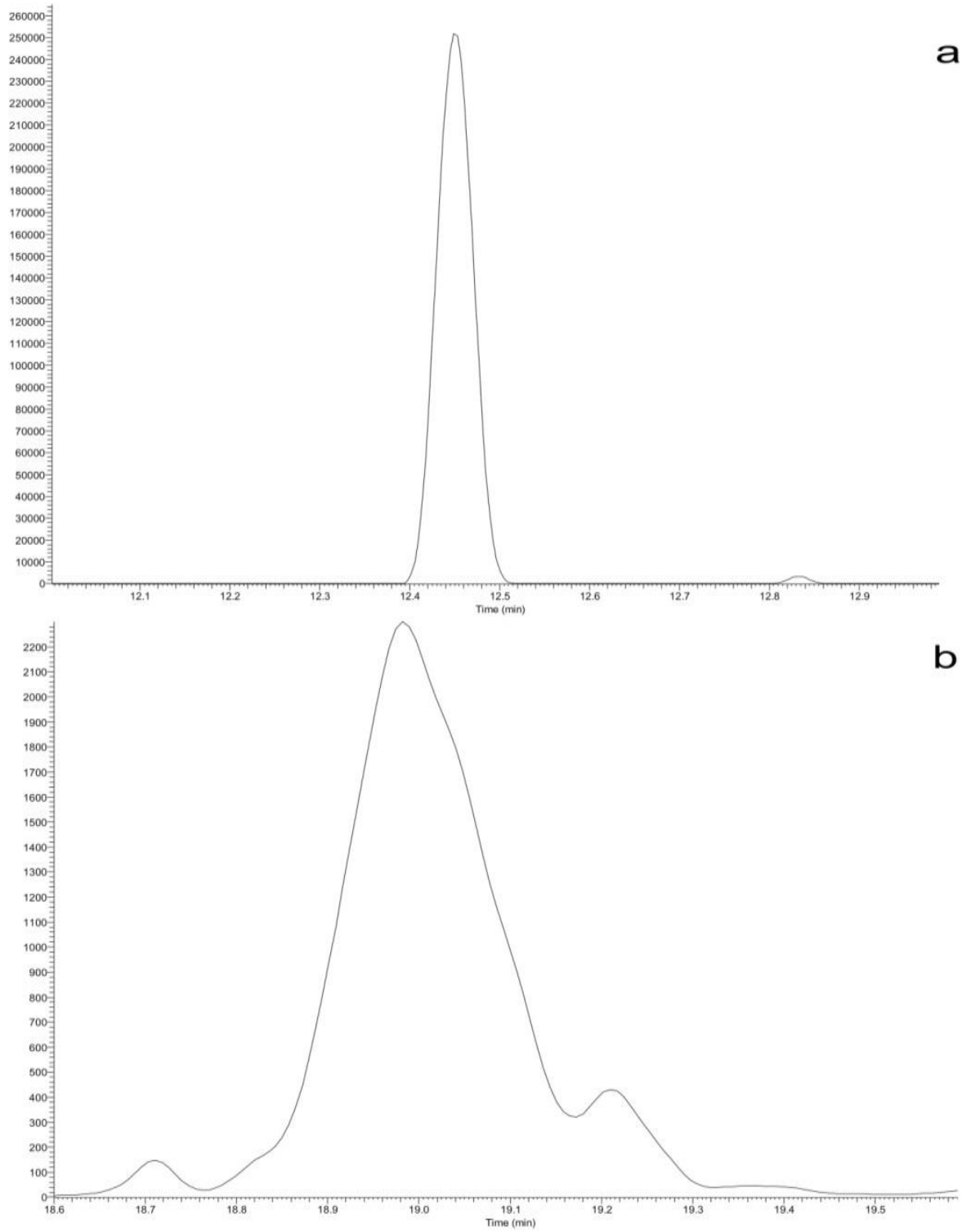

Figure 44: EIC of signature peptide <u>NLLGLIEAK</u> (485.8^{2+}) of a) 300 attomoles ProGRP in the developed manual LC-MS-system, and b) 2500 attomoles in the already established method [93].

6.2.6 OTER enzymes

To find the best suited enzyme for efficient protein digestion, OTERs were immobilized with Lys-C, trypsin and T/L, respectively (as described in the experimental section). Length of the reactor, temperature of digestion, and digestion time were also examined. An attempt to make a monolithic reactor was done (presented in Appendix 2, section 10), with the intention to compare it with the OTERs. However, time did not allow such comparison in the present study.

It should be noted that protein Mix I, used in the following experiments, contained the same mass concentration of each protein (5 µg/mL, Table 12) when evaluating the effect of length of OTERs (section 6.2.7) and the comparison of trypsin and T/L immobilized OTERs (section 6.2.8). In the experiments carried out later, protein Mix II (Table 13), with same molarity of each protein (0.000427 moles/mL), was used.

For the following experiments, OTERs prepared by the author were tested under the supervision of Hanne K. Hustoft.

Digestion efficiency of Lys-C, trypsin and T/L immobilized OTERs

As mentioned in the introduction, the most common enzymes used in proteomics are trypsin and Lys-C.

A schematic presentation of the chromatographic system used to evaluate the effect of the different enzymes immobilized into the OTERs is shown in Figure 45.

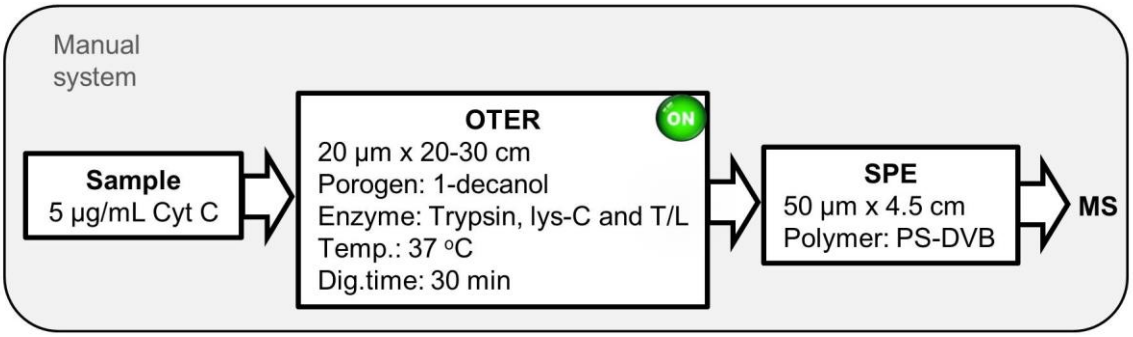

Figure 45: Highlights of the manual LC-MS-system that was used to evaluate the effect of different enzymes immobilized into the OTERs.

SQ % was used to evaluate the digestion efficiency by three injections of 5 µg/mL Cyt C, see Figure 46. The T/L immobilized OTER was 20 cm x 20 µm ID and gave average SQ % of 72 % of Cyt C. The trypsin immobilized OTER was 26 cm x 20 µm ID and could digest and identify 51 % of the amino acid sequence of Cyt C. The Lys-C immobilized OTER was 30 cm x 20 µm ID and gave an average SQ % of 25 % of Cyt C. Hence, the T/L immobilized reactor delivered more repeatable SQ % compared to the two other OTERs (data shown in Table 14 in Appendix).

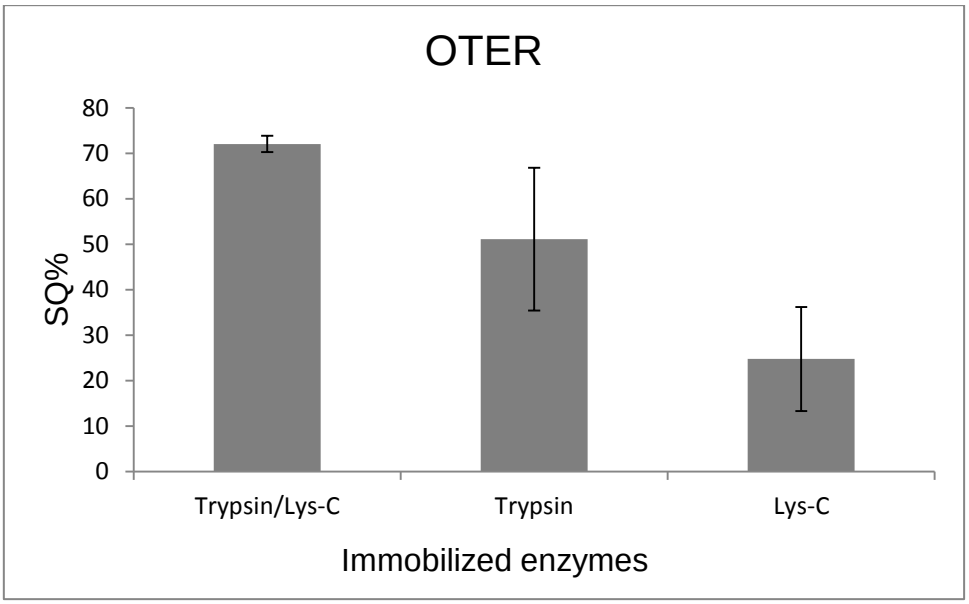

Figure 46: SQ % of 5 µg/mL Cyt C injected on short (20-30 cm) T/L, trypsin and Lys-C immobilized OTERs (n = 3).

6.2.7 Effect of OTER volume

Since T/L gave the best SQ % by on-line digestion of Cyt C, T/L was chosen as enzyme immobilized into the OTERs for this study. The highlights in the chromatographic system for testing different volumes of OTER are shown in Figure 47. Two 20 µm ID x 100 cm OTERs (prepared with 1-decanol) were possible to produced. However, in many attempt of reproducing long OTERs with 1-decanol as porogen was never achieved in later experiments. Therefore, it can only be concluded that up to 30 cm OTERs were reproducibly prepared.

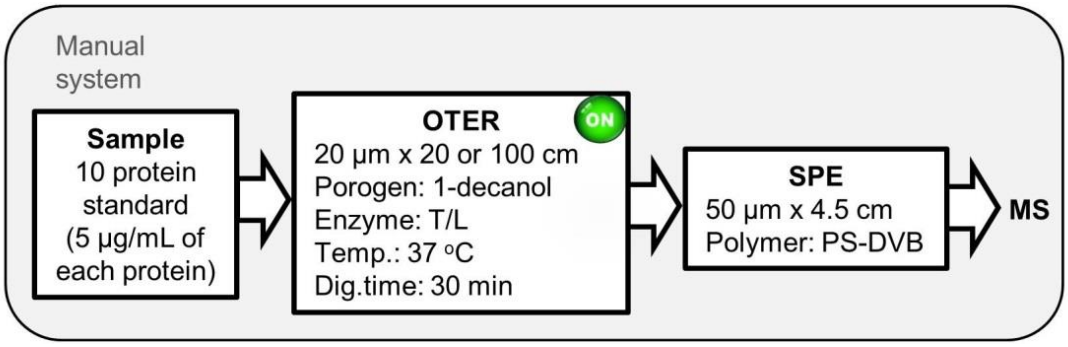

Figure 47: Highlights of the chromatographic system for testing volume of OTERs. A 20 µm x 20 cm OTER corresponds to a volume of 50 nL, while 20 µm x 100 cm OTER corresponds to a volume of 250 nL.

Protein Mix I (Table 12) was injected into two OTERs (0.8 µm polymer thickness) of different volume (50 and 250 nL). With the 50 nL OTER only two proteins were identified, while seven proteins were identified with the 250 nL OTER (Figure 48, data shown in Table 15 in Appendix). Six times more unique peptides were identified with the 250 nL OTER compared to the 50 nL OTER, see Figure 65 in Appendix.

This means that more proteins can be digested into peptides as the OTER becomes longer, i.e. higher enzymatic activity due to larger amount of immobilized enzymes. Ten out of ten proteins were identified later in the study (Figure 60) when injecting the same number of proteins on an OTER prepared with 1-heptanol as porogen (see section 6.4.2).

Hence, since the 250 nL OTER could digest far more proteins and gave better SQ % compared to the 50 nL OTER, the long OTER was therefore chosen in the future experiments.

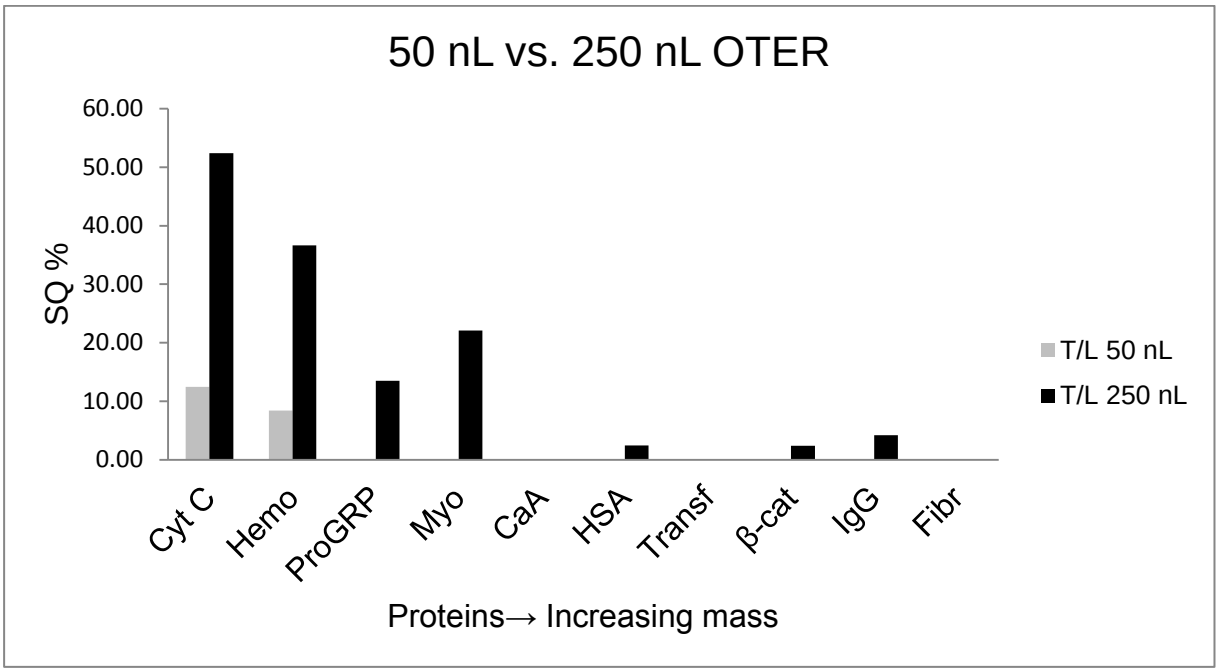

Figure 48: SQ % of a mixture of 10 proteins obtained on 50 nL and 250 nL OTERs (prepared with 1-decanol).

6.2.8 Comparison of trypsin and T/L immobilized 250 nL OTERs

The difference in SQ % provided by OTERs immobilized with trypsin and OTERs immobilized with T/L was marginal, see section 6.2.6. Since longer OTERs digested more proteins, see section 6.2.7, possible differences between trypsin and T/L was reinvestigated using 250 nL OTERs (prepared with 1-decanol). Figure 49 presents the main components of the chromatographic system used to compare 20 µm ID x 100 cm OTERs (250 nL) immobilized with T/L and trypsin, respectively.

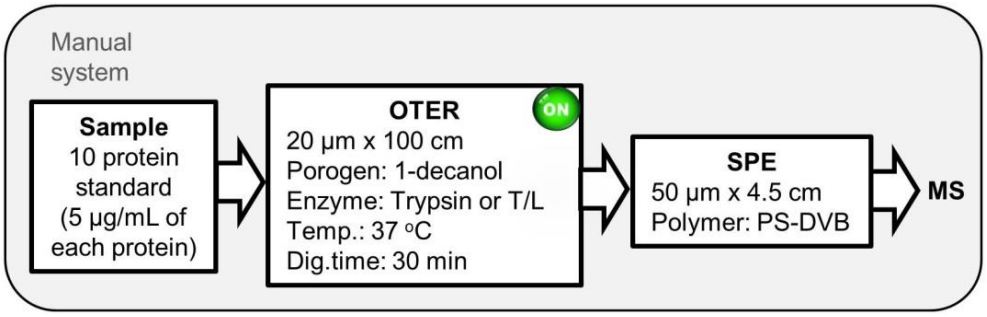

Figure 49: Highlights of the chromatographic system that was used to compare 250 nL trypsin and T/L immobilized OTERs.

Protein Mix I (Table 12), was used for the comparison. The SQ % was similar for trypsin and T/L (Figure 50), but T/L could digest more proteins, and also some of those with high Mm (data shown in Table 16 in Appendix). Thus, since both short and long T/L immobilized OTERs showed better performance than trypsin alone, T/L was chosen for further method development.

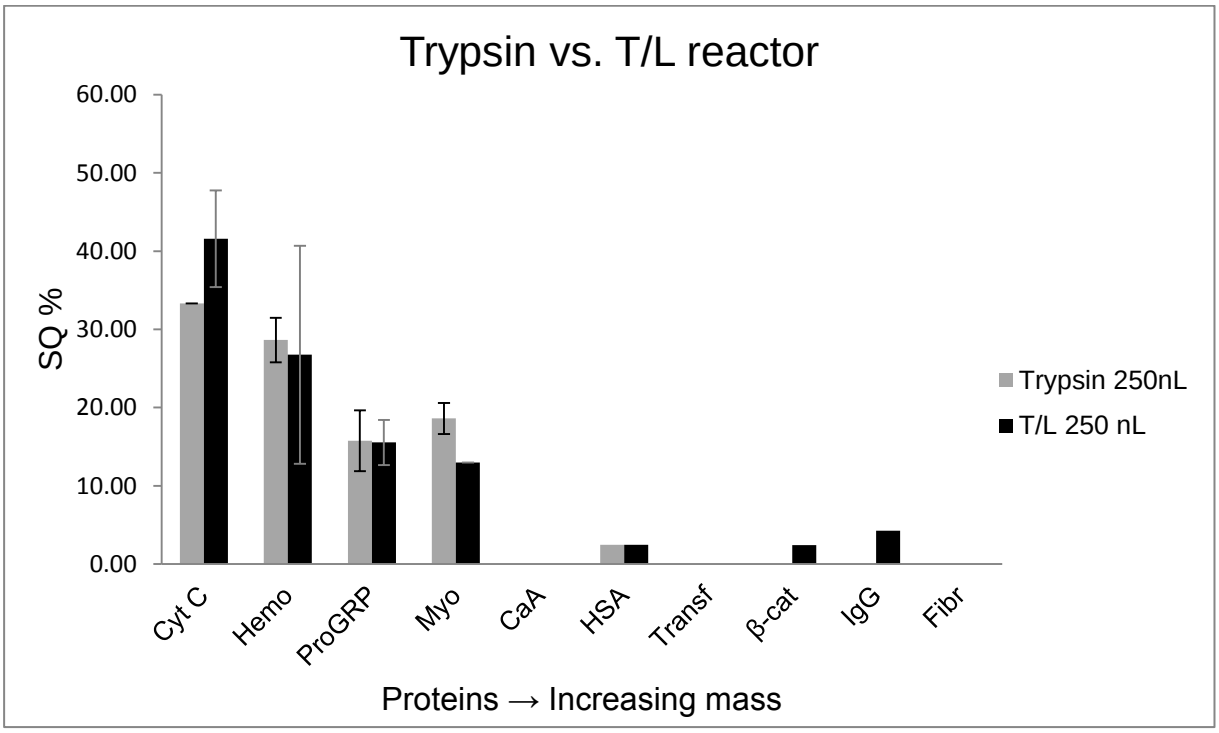

Figure 50: SQ % of proteins in a mixture of 10 proteins digested in 250 nL trypsin and T/L immobilized OTERs (n = 3).

6.2.9 Effect of temperature on the OTER digestion efficiency

A 250 nL T/L immobilized OTER was utilized for examining the effect of temperature on the protein digestion, because previous results (section 6.2.7 and 6.2.8) showed that long OTERs and T/L as enzymes for immobilization, give the best SQ %. The highlights of the chromatographic system used to evaluate the effect of temperature on the OTER during digestion are shown in Figure 51.

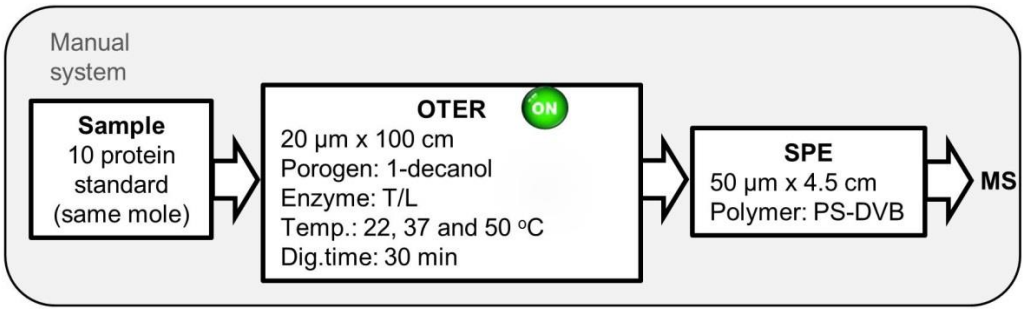

Figure 51: Highlights of the chromatographic system that was used to evaluate different temperatures on the OTER during digestion. This experiment was performed under supervision of Hanne K. Hustoft.

Using the manual LC-MS-system, on-line digestion of protein Mix II (listed in Table 13 in Appendix) was evaluated at 22, 37, 50 °C and a temperature gradient, see Figure 52 (data shown in Table 17 in Appendix). On-line digestion at 50 °C could digest one more protein compared to digestion at 22 °C, but the SQ % was best for 37 °C and 50 °C.

Initially, digestion of the protein mixture at constant temperatures was performed. After analyzing the results, a hypothesis was established regarding the proteins' ease of digestion at specific temperatures. Based on the statement of Turapov et al. [58], that a temperature gradient could digest proteins more efficiently, a gradient running from 22 to 50 °C was examined. As seen from Figure 52, the SQ % of the proteins with lower Mm was poorer when digestion was performed during a temperature gradient program (data shown in Table 18 in Appendix) compared to the digestions at the constant temperatures.

A digestion temperature of 37 °C was chosen for upcoming experiments, because 50 °C was less robust when longer digestion times were used, see section 6.2.10.

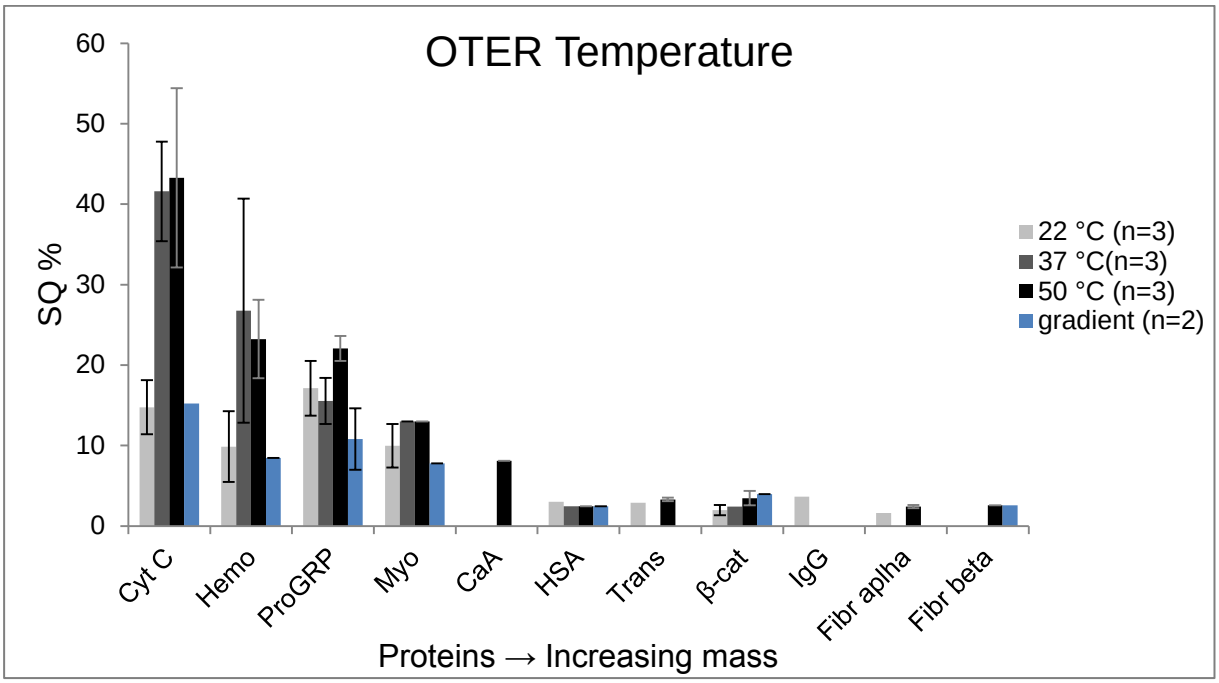

Figure 52: SQ % while keeping the OTER at different temperature during digestion. For the evaluation, 3 replicate injections (n) were used, except for the gradient which had only n = 2. The OTER was immobilized with T/L and could digest 250 nL of sample. Digestion time was 30 min for all injections.

6.2.10 Effect of digestion time

A 250 nL T/L immobilized OTER was used to evaluate if more protein could be digested if the protein Mix II (Table 13) was in the reactor for a longer period of time. The highlights of the chromatographic system used for this evaluation is shown in Figure 53. The digestion temperature was 50 °C and the digestion times were 5, 15, 30, 60 and 120 min.

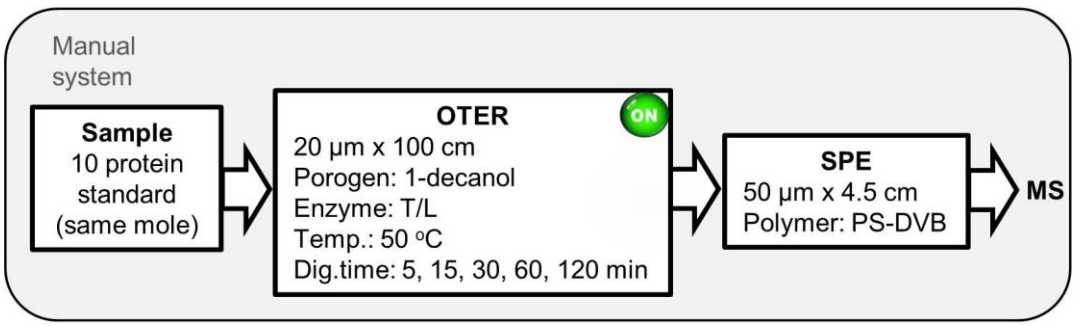

Figure 53: Highlights of the chromatographic system that was used to evaluate the effect of different digestion times.

Out of 10 proteins, 8 proteins were identified with 15 min digestion compared to 6 proteins with 5 min, see Figure 54 (data shown in Table 19 in Appendix). Seven proteins were identified with 30 min, while 8 and 4 proteins were identified with 1 and 2 h, respectively. Overall, the SQ % was quite similar. It was peculiar that fewer proteins were identified at longer digestion time, when actually the opposite was expected. A digestion time of 15 min seemed as the digestion time that gave the highest amount of identified proteins.

However, digestion while the gradient program was running (30 min) was chosen because this was more practical with regard to switching of the valves in the automated system when standard proteins were analyzed.

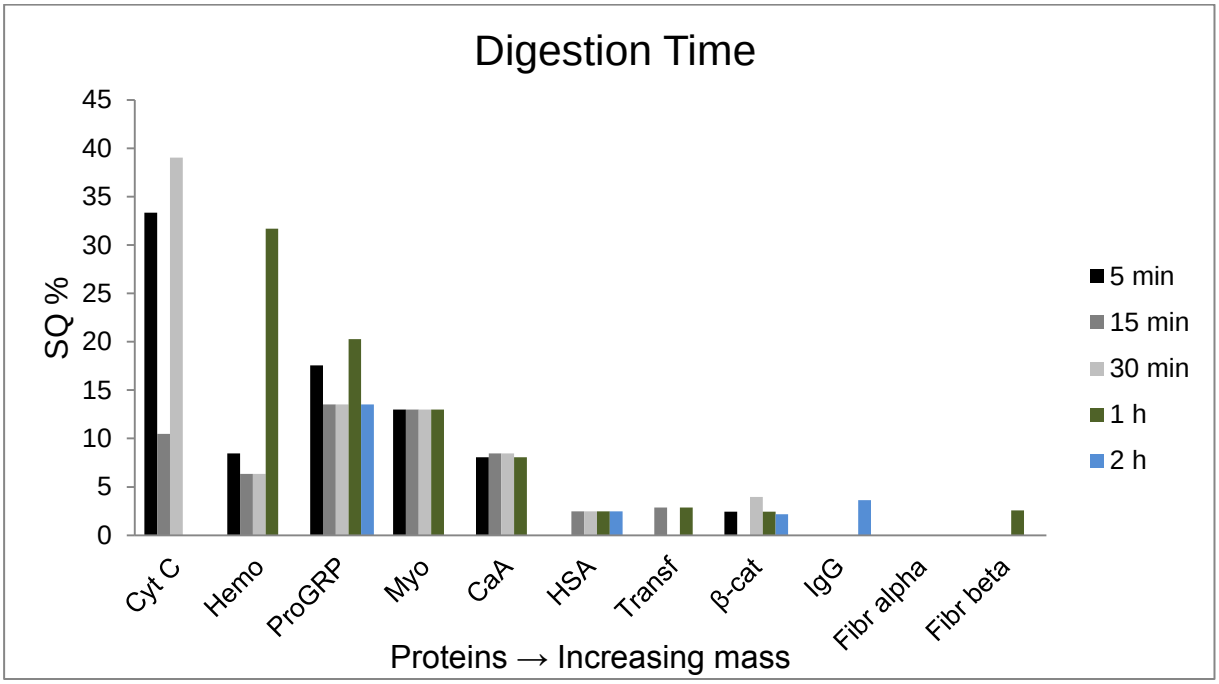

Figure 54: SQ % of mixture of 10 proteins (n = 1) for different digestion times (5 min, 15 min, 30 min, 1 h and 2 h).

6.3 Novel high capacity OTERs

6.3.1 Polymer thickness

In order to achieve a thicker polymer layer inside the OTER, and thus possibly immobilize more enzymes on the polymer layer compared to the original recipe, a crosslinker (EDMA) was added to the polymerization solution. As explained by Svec et al. [33], the internal structure begins to "construct" numerous interconnected pores when a crosslinker is added to the polymerization solution. This can also be observed in Figure 55 (column B, Table 6). The large globules produced by the crosslinker resulted in a high back-pressure for the column, which was not suitable for use in the on-line system. We were not able to find suitable conditions that would results in a layer exceeding 0.8 μm with even thickness.

When increasing the ID, from 20 to 50 μm ID, a non-uniform bulky structure of the polymer was observed (column C, Figure 55, Table 6). This column was not usable as it had to be 4 cm in order to be able to dry it with N_2 due to high back-pressure. According to Moravcova et al. [107], this might be due to radial temperature gradients formed across the diameter of the column during the exothermic polymerization process. This radial temperature gradient may also form void spaces in the column bed. He et al. found that going from 5 to 50 μm ID the column transformed from OT to monolithic column [108]. However, in the present study it is strange that a monolithic structure is formed since a crosslinker is not present.

Finding a recipe for the production of a monolithic HEMA-VDM column was attempted. This was done as a side project and the results are summarized in Appendix section 10.

6.3.2 Test of different porogens

As the thickness of the polymer layer was difficult to increase, different porogens in the polymerization solution was investigated in hope of being able to produce longer OTERs so that the enzymatic activity of the column could be increased. The different porogens used were 1-decanol, toluene, 1-cyclohexanedimethanol and 1-heptanol. They were added to the polymerization solution in the same ratio, see Table 7. The two capillaries that were polymerized with toluene and 1-cyclohexanedimethanol as porogens were not open (dense monolithic structure) after polymerization and could not be characterized further. The

capillary that was polymerized with 1-heptanol as porogen was open together with the capillary that was polymerized with 1-decanol.

Table 6: Polymerization solution for 20 µm ID (A, B, D) and 50 µm ID (C) capillaries with respect to SEM images in Figure 55. When the chemical was not added to the polymerization solution is indicated with "-" in the table.

	Column A*	**Column B**	**Column C**	**Column D**
ID (µm)	20	20	50	20
HEMA (g)	0.1200	0.1200	0.1200	0.1200
VDM (g)	0.0800	0.0800	0.0800	0.0800
1-decanol (g)	0.6000	0.6000	0.6000	-
AIBN (g)	0.0003	0.0003	0.0003	0.0003
EDMA (g)	-	0.1000	-	-
1-heptanol	-	-	-	0.6000

*original composition used by Huang et al. [97].

Table 7: Overview of the polymerization solution prepared when investigating the effect of different porogens.

Porogen / **Reagent**	1-decanol	toluene	1-cyclohexane-dimethanol	1-heptanol
HEMA (g)	0.08	0.08	0.08	0.08
VDM (g)	0.02	0.02	0.02	0.02
Porogen (g)	0.60	0.60	0.60	0.60
AIBN (g)	0.0001	0.0001	0.0001	0.0001
Open/closed After polymerization	Open	Closed	Closed	Open

The column polymerized with 1-heptanol as porogen was studied by SEM (column D, Figure 55, Table 6). A smoother surface was observed when using 1-heptanol instead of 1-decanol as porogen. The thickness of the wall became thinner and the globules smaller. Viklund et al. [35] states that larger globules are formed when poorer solvent for monomers are used. The comparison of column A and D (Figure 55, Table 6), suggests that 1-heptanol is a better solvent for the monomers, HEMA and VDM, compared with 1-decanol.

He et al. compared the use of 1-decanol and 1-octanol as porogen in the polymerization solution and tested the effect of polymerizing with the same polymerization solution in 50, 20, 10 and 5 μm ID capillaries [108]. They found that using an alcohol with a shorter carbohydrate chain, as porogen, gave a thinner film thickness and smaller globules.

In the present study, going from 1-decanol to 1-heptanol undoubtedly resulted in smaller globules compared to the column polymerized with 1-decanol. The thinner film thickness obtained also resulted in lower back-pressure, and hence longer OTERs could be prepared. The polymeric film thickness on the capillary wall of the OTER prepared with 1-heptanol was ~0.4 μm.

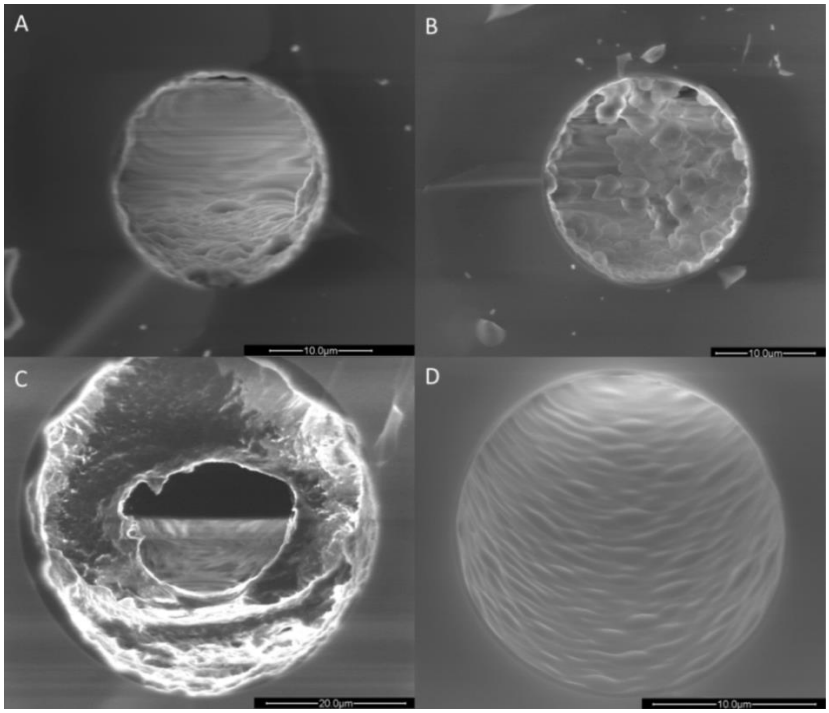

Figure 55: SEM images of different attempts of making OTER in 20 (A, B and D) and 50 μm ID (C). SEM image was taken using a LFD in low vacuum and a high voltage ranging from 8 - 14 kV.

6.3.3 Producing longer OTERs

When using 1-decanol as porogen, columns between 20 and 30 cm were successfully prepared, with the exception of two 100 cm OTERs (used in section 6.2.8, 6.2.9 and 6.2.10). OTERs with dimension 20 μm ID x 20 cm resulted in a total volume of ~50 nL when the film thickness inside this OTER was ~0.8 μm (Figure 56). Typically, in an attempt of

polymerizing ten 20 µm ID x 20 cm OTERs with 1-decanol as porogen, about six of these columns were open. Out of the six columns that were successfully polymerized, all six columns were also immobilized with enzyme and were able to digest protein standards. The limiting step for successful OTER production was found to be the polymerization step. Hence, the production of 20 cm long OTERs using 1-decanol as porogen was not completely flawless.

In comparison to the 20 cm OTER prepared with 1-decanol, the 20 cm OTER prepared with 1-heptanol as porogen had a film thickness of ~0.4 µm (Figure 56), and contain therefore a volume of ~60 nL. The production of OTER was easier with 1-heptanol as porogen, and up to 150 cm long OTERs were easily prepared. Hence, the 150 cm OTERs could contain a volume of ~350 nL, which means it had a larger surface area and could digest larger sample sizes. The ease of production with 1-heptanol as porogen was confirmed by the fact that ten out of ten columns being open after the polymerization step. These ten columns were also successfully immobilized after the polymerization step and could digest the protein standard.

It was important that long OTERs could be prepared, and installed into the LC-MS-system, since they have a larger surface area that is a step closer to the surface area of monolithic IMERs.

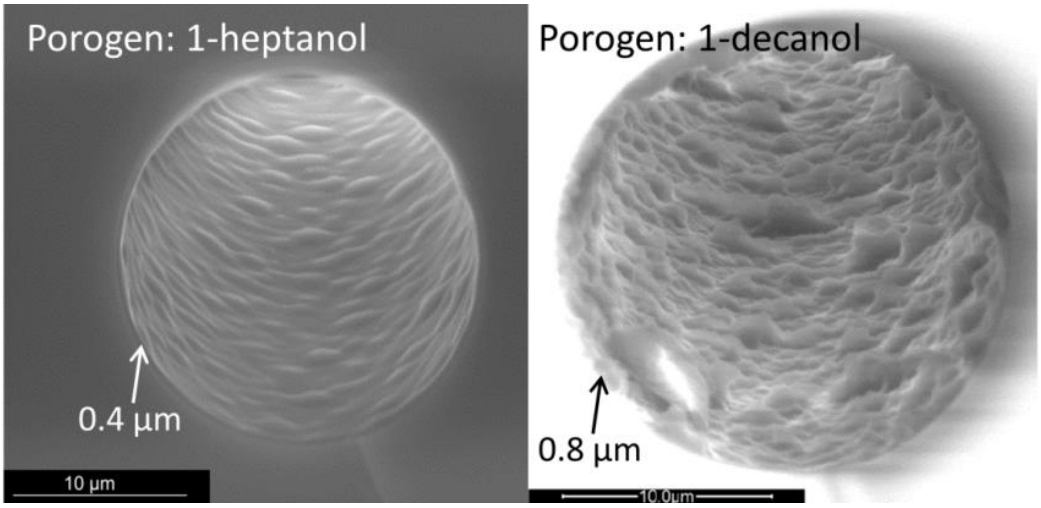

Figure 56: SEM images of 20 µm ID OTER prepared with 1-heptanol (left) and 1-decanol (right) as porogen. The configuration of the SEM in order to be able to take the images was low vacuum, with LFD, and high voltage 14 kV.

6.4 Use of novel OTER in the automated LC-MS-system

The developed system was automated by Tore Vehus, and the developed OTER was integrated in this system. The author contributed to the experiments, under the supervision of Hanne K. Hustoft. The results have been addressed in the manuscript by Hustoft et al. [71].

An injection replicate in the manual system took about 60 min (digestion and analysis), while the automated system used 240 min. However, there are far more advantages with the automated LC-MS-system compared to the manual LC-MS-system. The manual system requires an operator switching the vales at the correct time. If the valves are not switched at the exact same time from run to run, then the repeatability of the system will not be satisfying. An automated system is more repeatable, there is less maintenance required and there are far less requirements to the operator. In addition, an automated LC-MS-system can analyze samples 24/7.

In order to evaluate the automated system a mixture of 10 proteins was digested in-solution and analyzed (Appendix section 12). Carry-over of the automated OTER-SPE-PLOT LC-MS-system was examined and the applicability of the system for complex samples was shown using an injection of a human cell lysate.

6.4.1 Quenching vs. not quenching

Huang et al. recommended to quench the columns after immobilization with ethanolamine in order to omit carry-over issues [97]. However in the present study, it was found that clogging occurred during quenching, and the columns (prepared with 1-decanol as porogen) were not always usable after quenching. As ethanolamine is highly corrosive, it is possible that some of the larger globules were etched off the column wall and clogged the column. Therefore OTERs made with 1-decanol were not quenched in our laboratory.

When 1-heptanol was implemented in the polymerization solution, the OTERs were possible to quench. A comparison of digestion efficiency of an OTER prepared with 1-heptanol that was quenched and one that was not quenched for protein Mix II (Table 13). Figure 58 (data shown in Table 8) shows that there is clearly better SQ % with the column that was not quenched. Therefore this step in the column preparation was taken out of the process, and the columns were ready to use right after immobilization. The columns were always flushed with

50 mM NH_4OAc after immobilization so that the enzyme solution was not in the column during storage.

A schematic illustration of the chromatographic system used to test quenched vs. not quenched OTERs is shown in Figure 57. For unknown reasons, Cyt C was not identified from the protein standard mixture when comparing an OTER that was quenched and one that was not quenched. In addition, these results confirm that the SPE column serve its purpose of retaining sufficient peptides for identification of proteins.

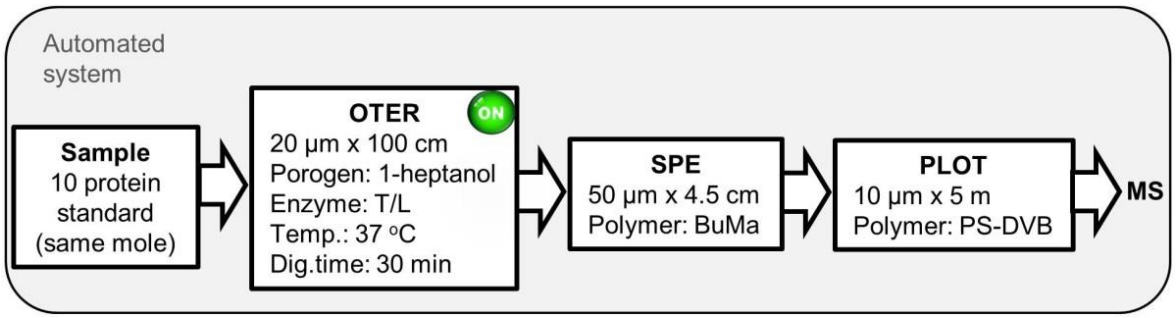

Figure 57: Schematic illustration of the highlights in the chromatographic system that was used for testing quenched vs. not quenched OTER.

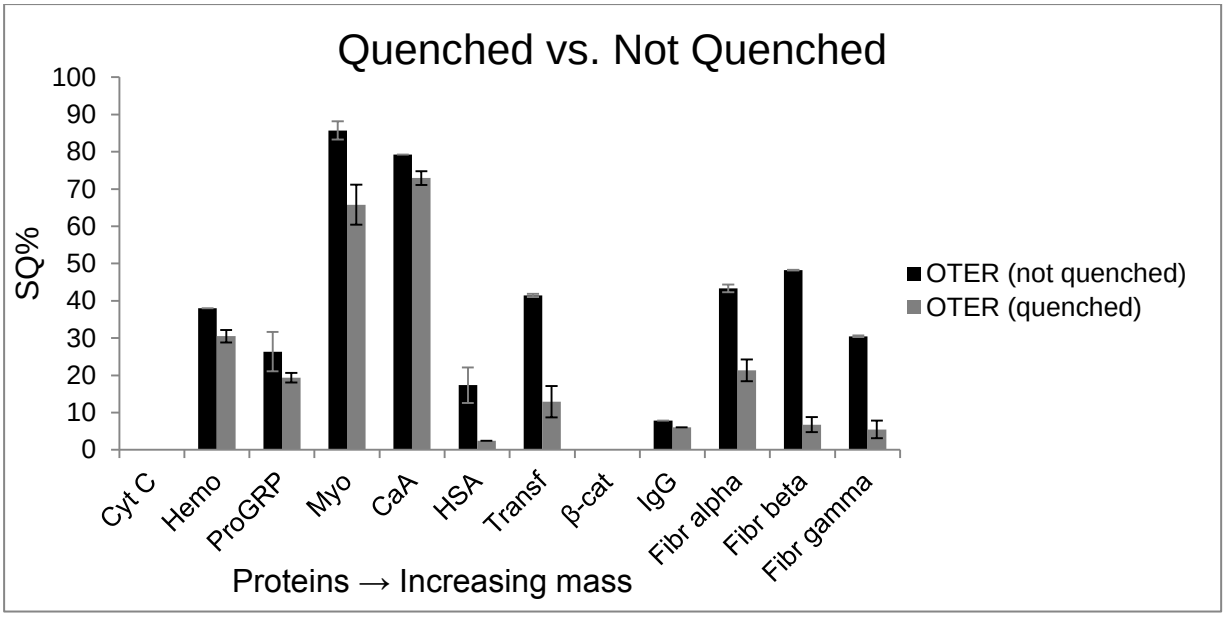

Figure 58: SQ % of mixture of 10 standard proteins on 20 µm ID OTERs (290 nL) quenched and not quenched with ethanolamine. The automated system was used for this comparison with OTER-SPE-PLOT (n = 3).

To sum up, it was found that preparing the OTERs with 1-heptanol as porogen instead of 1-decanol gave a more repeatable production. Longer OTERs could also be made with the new porogen. Quenching was not performed in the OTERs, because it lowered the average SQ % of the proteins, even though quenching of the OTERs has been recommended [97].

6.4.2 Within and between OTER digestion repeatability

The 290 nL developed OTER polymerized with 1-heptanol was tested using the automatic system. The highlights in the chromatographic system are shown in Figure 59.

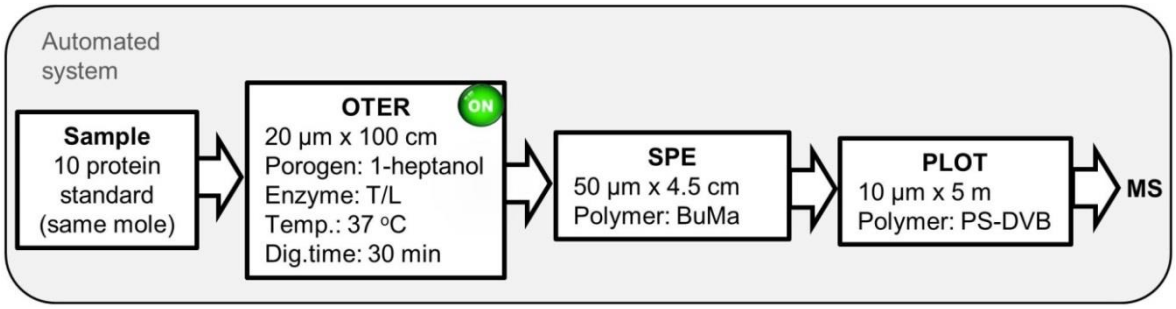

Figure 59: Highlights in the chromatographic system that was used to evaluate the within and between OTER digestion repeatability. A 20 µm ID x 100 cm OTER prepared with 1-heptanol corresponds to 290 nL volume. The experiments were performed under the supervision of Hanne K. Hustoft.

Three 290 nL OTERs were each used for three replicate injections of protein Mix II (Table 13) which were digested on-line for 30 min. SQ % obtained for these proteins was used for performance evaluation; see Figure 60 (data shown in Table 20 in Appendix).

All the proteins in Mix II were digested (10 out of 10) and identified by the 290 nL OTERs prepared with 1-hepanol as porogen, and they showed good repeatability as discussed in the manuscript by Hustoft et al. [71]. St. Dev. was between 0 and 4 for most proteins. However, a St. Dev. of 11 was found for Cyt C in OTER 2.

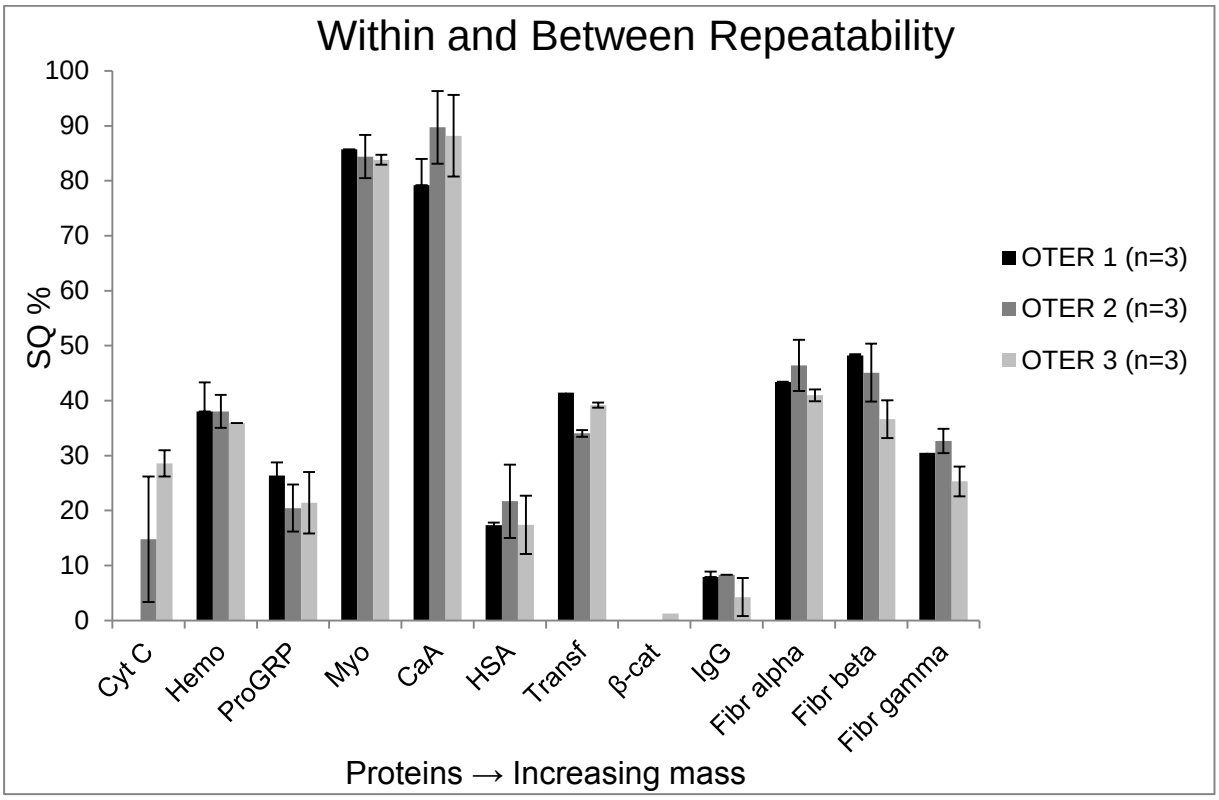

Figure 60: SQ % of protein mixture using three 290 nL OTERs immobilized with T/L. Each OTER was tested by on-line digestion with three replicate injections of the mixture of 10 proteins of equal molarity (n = 3, Error bars are calculated for within OTER repeatability).

From Figure 60, it can be concluded that the variation for the within and between repeatability is expected for in-house prepared columns. This also confirms that the preparation of OTERs with 1-heptanol as porogen is repeatable, which is also the case for other open tubular columns, as mentioned earlier.

6.4.3 Carry-over in the automated LC-MS-system

For reliable analysis, low carry-over is required. The manual system showed an average carry-over below 1.6 %. However, since the tubings and valves used in the automatic system were different, carry-over had to be tested in the automatic system.

A carry-over test was carried out in the automated system in order to be compared to the manual system (see Section 6.2.4). The highlights of the chromatographic system used for this evaluation is shown in Figure 61.

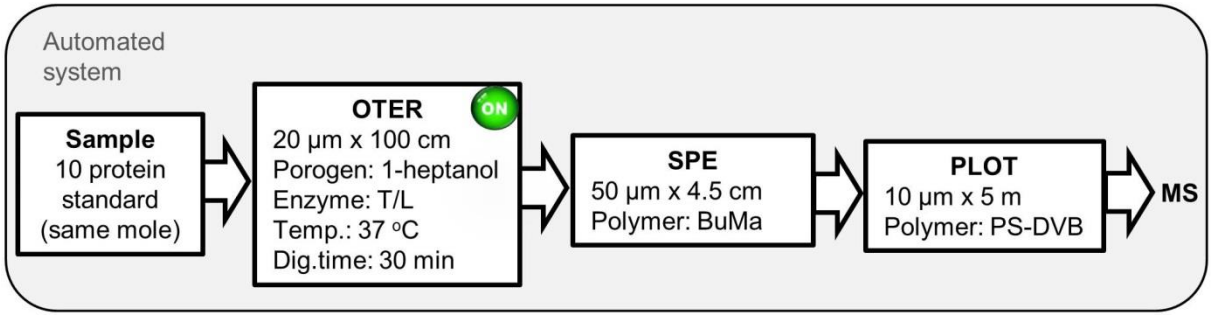

Figure 61: Highlights in the chromatographic system used for the carry-over test in the automated LC-MS-system.

The carry-over in the automated LC-MS-system was evaluated by injection of protein Mix II (Table 13). The EIC of the most abundant peaks in the TIC from the analysis of the 10 protein mixture was compared with the following blank injection. In the blank following the 10 protein mixture injection, none of the peaks from the EIC could be found (Figure 62). Thus, the carry-over was low in the automated chromatographic system. However 2.5 % carry-over was observed for some peptides when injecting high amount of proteins (75 µg). These results have been addressed in the manuscript by Hustoft et al. [71].

Compared to the manual system (section 6.2.4), the automatic system showed even lower carry-over. Better "washing" of the tubings and columns after each injection might be an explanation to this. Hence, the system was ready for a new sample injection after each injection.

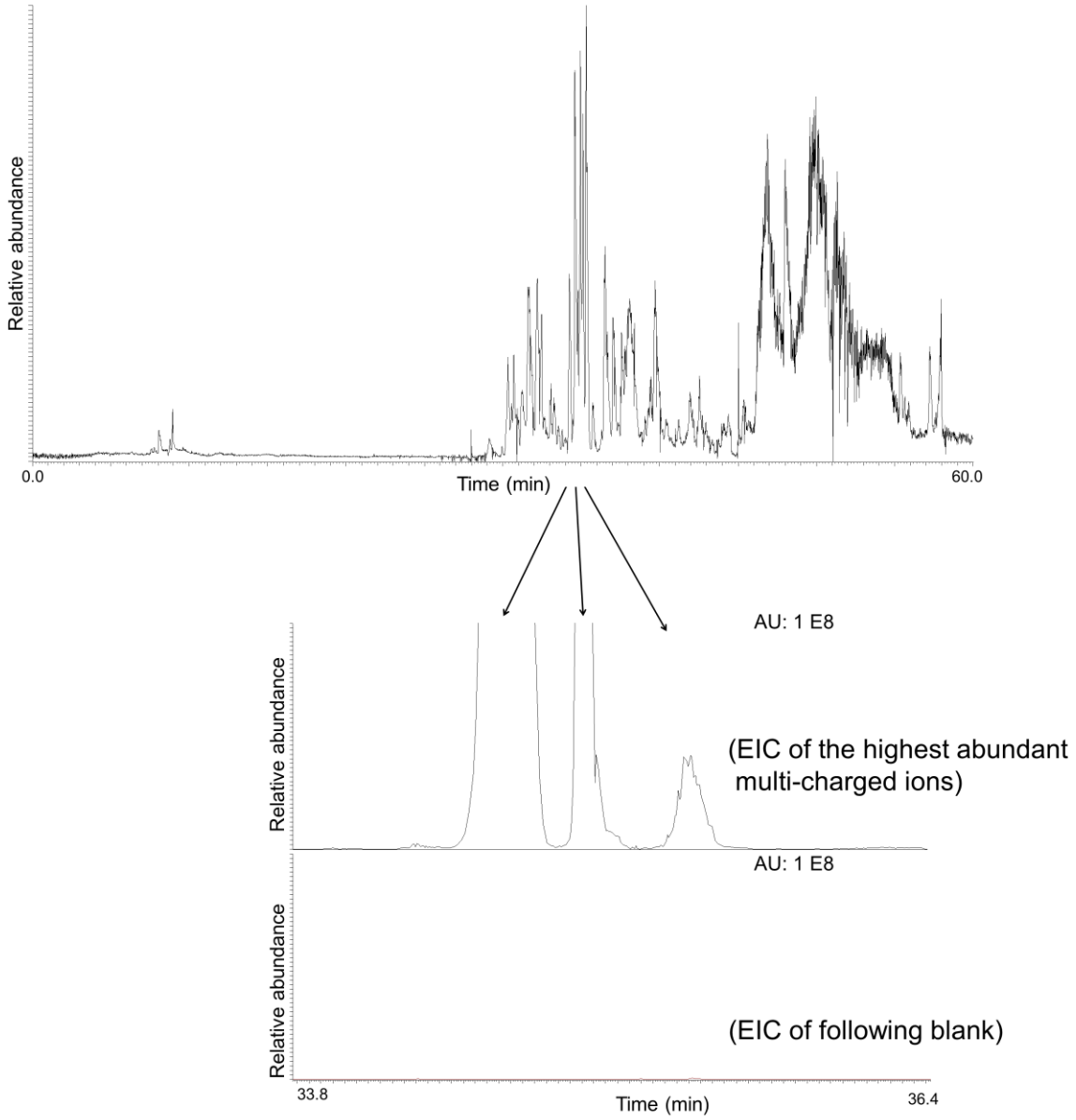

Figure 62: Carry-over in the automated LC-MS-system tested by injecting Mix II. Upper chromatogram: TIC of the mixture of 10 proteins. Lower chromatograms: Zoom-in to the three highest abundant ions from the injection of 10 proteins (above) and zoom-in on the same ions in the following blank injection (below, not able to extract). AU is arbitrary units.

6.4.4 Analysis of real sample

A complex human cell lysate sample was received from Rikshospitalet in a concentration of 1 µg/µL, and analyzed by Tore Vehus using the chromatographic system shown in Figure 63. Four 1 m OTERs were coupled together so that they could take a sample volume of almost 1.2 µL, and the lysate sample was digested for 30 min before the chromatographic analysis. About 1.2 µg of human cell lysate was injected into the OTER. Almost 1500 proteins were identified (Figure 64) using high peptide confidence level (the most strict search parameter in Proteome Discover). The results have been further discussed in the manuscript by Hustoft et al. [71].

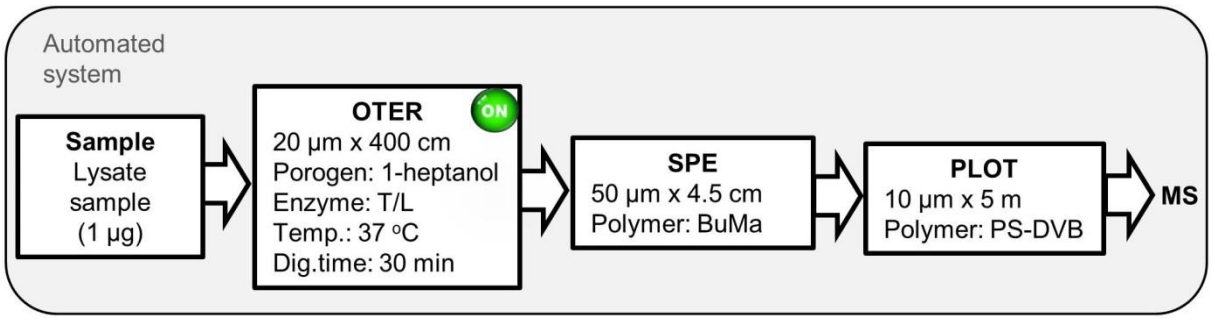

Figure 63: Highlights in the chromatographic system used for analysis of lysate sample. The experiments were carried out by Tore Vehus under the supervision of Hanne K. Hustoft.

In a review by Safdar et al. [92] other OT columns immobilized with enzymes are discussed. Among the discussed columns is a dextran coated capillary that was covalently immobilized with pepsin [78]. This reactor could digest human Hemo, Myo and bovine serum albumin in 3 min, which was impressive, but they did not use this reactor for more complex samples. Another OT reactor had trypsin adsorbed onto a sol-gel matrix [79]. This trypsin immobilized reactor could digest β-casein, Myo and Cyt C in impressive 24 seconds, and 253 proteins were identified in a single run of a real biological sample. The same article states that automation of the system is necessary in the future in order to perform proteome analysis.

Nagaraj et al. [109] reported in 2012 that they could observe almost 4000 yeast proteins when 140 µg of yeast proteins were loaded onto the filter. These proteins were identified in a 4 h long reversed phase LC-ES-MS/MS run when performing protein digestion using a filter-aided sample preparation (FASP) method [110] and a Orbitrap mass analyzer.

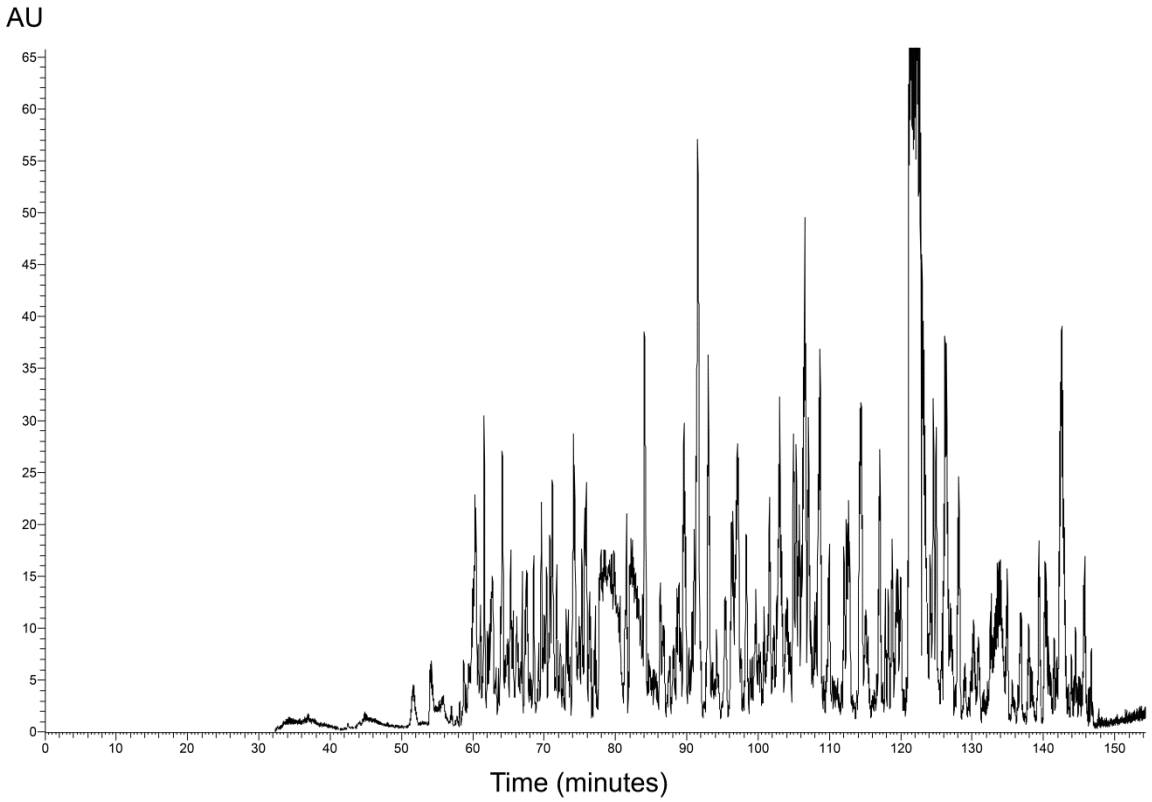

Figure 64: Full MS chromatogram of lysate sample received from Tore Vehus in concentration of 1 µg/mL.

7 Conclusions

An IMER in the open tubular format named OTER has been successfully developed in 20 μm ID fused silica capillaries for on-line digestion of proteins in a nano LC-MS based proteomic platform, both manual and automated. Short OTERs (30 cm) could be prepared with 1-decanol as porogen, while longer reactors (up to 150 cm) were achieved with 1-heptanol as porogen. Long OTERs immobilized with T/L were found to enhance the digestion efficiency. A short OTER integrated in the manual nanoproteomic platform could efficiently digest 300 attomoles of the target protein ProGRP, and even lower detection limits could be expected for the long OTER, which can handle larger sample sizes. Both the manual and the automated system could run samples subsequently due to low carry-over in the system. The OTERs showed good within and between digestion repeatability in the automated system. The automated system allowed sample analysis 24/7 and has fewer demands to the operator. About 1500 proteins were identified in a real human cell lysate sample using on-line digestion with the long OTER.

This shows the great potential of this OTER in the automated on-line digestion nano LC-MS proteomic platform, for fast analysis of biological samples (of limited size) both in comprehensive and targeted proteomics.

8 References

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9 Appendix 1

Raw data has not been adjusted to the correct number of significant digits.

9.1 Additional tables

Table 8: SQ % of mixture of standard proteins from comparison of quenched and not quenched OTER. It should be noted that Cyt C was not found in this protein standard.

OTER (not quenched)	Rep 1	Rep 2	Rep 3	Average	St. dev
Cyt C					
Hemo	38.03	38.03	38.03	38.03	0.00
ProGRP	20.27	25.68	33.11	26.35	5.26
Myo	88.96	83.12	85.06	85.71	2.43
CaA	79.23	79.23	79.23	79.23	0.00
HSA	18.23	11.17	22.66	17.35	4.73
Transf	40.83	41.83	41.69	41.45	0.44
β-cat					
IgG	7.88	7.88	7.88	7.88	0.00
Fibr alpha	44.72	43.09	42.28	43.36	1.02
Fibr beta	48.08	48.29	48.29	48.22	0.10
Fibr gamma	30.63	30.18	30.63	30.48	0.21
OTER (quenched)	**Rep 1**	**Rep 2**	**Rep 3**	**Average**	**St. dev**
Cyt C					
Hemo	31.69	31.69	28.17	30.52	1.66
ProGRP	17.57	20.27	20.27	19.37	1.27
Myo	73.38	61.69	62.34	65.80	5.36
CaA	70.38	74.62	73.85	72.95	1.84
HSA		2.46		2.46	0.00
Transf	14.61	7.16	17.05	12.94	4.21
β-cat					
IgG	6.06			6.06	0.00
Fibr alpha	17.72	21.46	24.88	21.35	2.92
Fibr beta	4.06	8.97	7.26	6.76	2.04
Fibr gamma	3.83	3.83	8.78	5.48	2.33

Table 9: Relative peak area of four extracted peptides (*m/z* 908.46, 851.69, 943.02 and 460.29) as a function of trapping time into SPE column (1, 2.5, 4 and 6 min), i.e. the time from the OTER to the SPE column (PS-DVB), from injection of 2.5 µg/mL in-solution digested Myo into a 20 µm ID x 15 cm OTER immobilized with trypsin. Results are presented in Figure 36.

1 min	**A(*m/z* 908.46)**	**A(*m/z* 851.69)**	**A(*m/z* 943.02)**	**A(*m/z* 460.29)**
2.5 µg/ml Myo Rep 1	65755906	17458344	35008666	371467494
2.5 µg/ml Myo Rep 2	6683964	2868937	5786868	35913406
2.5 µg/ml Myo Rep 3	32201001	6633149	6700373	32847233
2.5 µg/ml Myo Rep 4	68923007	40154885	37101761	170167612
Average	43390970	16778829	21149417	152598936
St. Dev.	29578680	16766252	17236965	159340398
2.5 min	**A(*m/z* 908.46)**	**A(*m/z* 851.69)**	**A(*m/z* 943.02)**	**A(*m/z* 460.29)**
2.5 µg/ml Myo Rep 1	32633304	6158471	12966510	155621603
2.5 µg/ml Myo Rep 2	91851616	27209864	42149875	323543987
2.5 µg/ml Myo Rep 3	66844756	4961154	26264900	355967136
2.5 µg/ml Myo Rep 4	20899736	12508850	26752536	185742172
Average	53057353	12709585	27033455	255218725
St. Dev.	32384880	10218507	11931117	99272624
4 min	**A(*m/z* 908.46)**	**A(*m/z* 851.69)**	**A(*m/z* 943.02)**	**A(*m/z* 460.29)**
2.5 µg/ml Myo Rep 1	98816387	33890218	90448366	285432295
2.5 µg/ml Myo Rep 2	167225402	23346551	124018044	703107744
2.5 µg/ml Myo Rep 3	80623365	13600126	33543587	588651382
Average	115555051	23612298	82669999	525730474
St. Dev.	45663064	10147656	45736026	215829738
6 min	**A(*m/z* 908.46)**	**A(*m/z* 851.69)**	**A(*m/z* 943.02)**	**A(*m/z* 460.29)**
2.5 µg/ml Myo Rep 1	43575243	8768019	44150052	363368810
2.5 µg/ml Myo Rep 2	74718955	12147125	64557682	621863498
2.5 µg/ml Myo Rep 3	39749035	5320920	18836890	279900286
Average	52681078	8745355	42514875	421710865
St. Dev.	19181006	3413159	22904215	178290651

Table 10: Relative peak area of four extracted peptides (*m/z* 908.46, 851.69, 943.02 and 460.29) as a function of % mobile phase B in the loading buffer (1, 4 and 10 %), i.e. the loading buffer from the OTER to the SPE column, from injection of 2.5 µg/mL in-solution digested Myo into a 20 µm ID x 15 cm OTER immobilized with trypsin. Results are presented in Figure 37.

1 % mobile phase B in loading buffer	**A(*m/z* 908.46)**	**A(*m/z* 851.69)**	**A(*m/z* 943.02)**	**A(*m/z* 460.29)**
2.5 µg/ml Myo Rep 1	53662123	14943023	34628449	273928436
2.5 µg/ml Myo Rep 2	46738833	5519622	20892553	242062155
2.5 µg/ml Myo Rep 3	41578838	5454490	18444190	223826380
Average	47326598	8639045	24655064	246605657
St. dev	6063048	5459502	8723527	25358166
4 % mobile phase B in loading buffer	**A(*m/z* 908.46)**	**A(*m/z* 851.69)**	**A(*m/z* 943.02)**	**A(*m/z* 460.29)**
2.5 µg/ml Myo Rep 1	98816387	33890218	90448366	285432295
2.5 µg/ml Myo Rep 2	167225402	23346551	124018044	703107744
2.5 µg/ml Myo Rep 3	80623365	13600126	33543587	588651382
Average	115555051	23612298	82669999	525730474
St. dev	45663064	10147656	45736026	215829738
10 % mobile phase B in loading buffer	**A(*m/z* 908.46)**	**A(*m/z* 851.69)**	**A(*m/z* 943.02)**	**A(*m/z* 460.29)**
2.5 µg/ml Myo Rep 1	56380663	8618221	45915826	620850599
2.5 µg/ml Myo Rep 2	54830839	14720716	32240266	418792985
2.5 µg/ml Myo Rep 3	48678104	12893269	39063300	485318138
Average	53296535	12077402	39073131	508320574
St. dev	4074054	3131987	6837785	102974045

Table 11: Area of EIC peak at *m/z* 485.8^{2+} from ProGRP with increasing concentration from 250 to 5000 ng/mL.

Concentration (ng/mL) ProGRP	**Area**
250	294980
500	434862
1000	565106
5000	1451418

Table 12: Protein Mix I; Overview of the concentration of each protein in the protein mixture used for evaluation of off-line digestion, effect of length of enzymatic reactor, effect of temperature on enzymatic reactor, and evaluation of the time the mixture was digested.

Size (Da)	Protein	µg/mL	mol/mL	mmol/mL	µmol/mL
11700	Cyt C	5	0.000427	0.43	427.35
15200	Hemo	5	0.000329	0.33	328.95
16200	ProGRP	5	0.000309	0.31	308.64
17100	Myo	5	0.000292	0.29	292.40
29100	CaA	5	0.000172	0.17	171.82
36100	IgG	5	0.000139	0.14	138.50
69300	HSA	5	0.0000722	0.07	72.15
77000	Transf	5	0.0000649	0.06	64.94
85400	Beta catenin	5	0.0000586	0.06	58.55
340000	Fibr	5	0.0000147	0.01	14.71

Table 13: Protein Mix II; Overview of number of µL of each protein taken from their respective stock solutions (1 mg/mL) to obtain the same molar concentration as Cyt C. A total of 300 µg protein was in this protein mixture.

Size (Da)	Protein	mg/mL	µL	µg/mL	mol/mL
11700	Cyt C	1	5.00	5.00	0.000427
15200	Hemo	1	6.49	6.50	0.000427
16200	ProGRP	1	6.92	6.92	0.000427
17100	Myo	1	7.31	7.31	0.000427
29100	CA 2	1	12.44	12.44	0.000427
36100	IgG	1	15.43	15.43	0.000427
69300	HSA	1	29.62	29.62	0.000427
77000	Transf	1	32.91	32.91	0.000427
85400	β-cat	1	36.50	36.50	0.000427
340000	Fibr	1	145.30	145.30	0.000427

Table 14: SQ % of 5 µg/mL Cyt C digested on-line in a 20 µm ID OTER immobilized with T/L, trypsin and Lys-C. Note that the OTERs were not of the same length. The data are presented in Figure 46.

cm	Enzyme immobilized into OTER	Rep 1	Rep 2	Rep 3	Average	St. Dev.
20	T/L	73.33	73.33	69.52	72.06	1.80
26	Trypsin	68.57	54.29	30.48	51.11	15.71
30	Lys-C	40.95	17.14	16.19	24.76	11.45

Table 15: Protein Mix I (Table 12) was injected into the 50 nL and 250 nL OTER (1-decanol as porogen) immobilized with T/L in order to evaluate length of the reactors. The SQ % of the 10 proteins are listed in the table, and presented in Figure 48.

		T/L 50 nL	T/L 250 nL
Mm	**Protein**	**SQ %**	**SQ %**
11.7	Cyt C	12.45	52.38
15.2	Hemo	8.45	36.62
16.2	ProGRP		13.51
17.1	Myo		22.08
29.1	CaA		
69.3	HSA		2.46
77	Transf		
85.4	β-cat		2.43
144	IgG		4.24
340	Fibr		

Table 16: SQ % of proteins used in the comparison of 250 nL trypsin and 250 nL T/L immobilized OTERs (1-decanol as porogen) by injecting protein Mix I (Table 12). The data are presented in Figure 50.

	Trypsin 250 nL					**T/L C 250 nL**				
	Rep 1	**Rep 2**	**Rep 3**	**Average**	**St. Dev.**	**Rep 1**	**Rep 2**	**Rep 3**	**Average**	**St. Dev.**
Cyt C	33.33	33.33		33.33	0.00	41.90	47.62	35.24	41.59	6.20
Hemo	28.17	31.69	26.06	28.64	2.84	16.90	36.62		26.76	13.94
ProGRP	20.27	13.51	13.51	15.76	3.90		13.51	17.57	15.54	2.87
Myo	20.78	16.88	18.18	18.61	1.99	12.99	12.99	12.99	12.99	0.00
CaA										
IgG							4.24		4.24	
HSA			2.46	2.46			2.46		2.46	
Transf										
β-cat							2.43	2.43	2.43	
Fibr										

Table 17: SQ % of three replicate injections of protein Mix II (Table 13) digested on-line in a T/L immobilized OTER (250 nL) kept at 22, 37 and 50 °C. The data are presented in Figure 52.

22 °C (n = 3)					
Protein	**Rep 1**	**Rep 2**	**Rep 3**	**Average**	**St. Dev.**
Cyt C	12.38		17.14	14.76	3.37
Hemo	6.34	8.45	14.79	9.86	4.40
ProGRP	13.51	20.27	17.57	17.12	3.40
Myo	7.79	12.99	9.09	9.96	2.71
CaA					
HSA	2.46		3.59	3.025	
Trans		2.87	2.87	2.87	
β-cat	1.54		2.43	1.99	0.63
IgG	3.64			3.64	
Fibr-alpha	1.63			1.63	
Fibr-beta					
37 °C (n = 3)					
Protein	**Rep 1**	**Rep 2**	**Rep 3**	**Average**	**St. Dev.**
Cyt C	41.90	47.62	35.24	41.59	6.20
Hemo	16.90	36.62		26.76	13.94
ProGRP		13.51	17.57	15.54	2.87
Myo	12.99	12.99	12.99	12.99	0.00
CaA					
IgG		4.24		4.24	
HSA		2.46		2.46	
Transf					
β-cat		2.43	2.43	2.43	
Fibr					
50 °C (n = 3)					
Protein	**Rep 1**	**Rep 2**	**Rep 3**	**Average**	**St. Dev.**
Cyt C	50.80	48.57	30.48	43.28	11.14
Hemo	20.42	20.42	28.87	23.24	4.88
ProGRP	20.27	22.97	22.97	22.07	1.56
Myo	12.99	12.99	12.99	12.99	0.00
CaA		8.08	8.08	8.08	0.00
HSA	2.46	2.46	2.46	2.46	0.00
Trans	3.46		3.15	3.30	0.22
β-cat	3.97	3.97	2.43	3.46	0.89
IgG					
Fibr-alpha		2.56	2.28	2.42	0.20
Fibr-beta	2.56		2.56	2.56	0.00

Table 18: SQ % of two replicate injections protein Mix I (Table 12), digested on-line with T/L immobilized OTER (250 nL) at increasing temperature from 22 to 50 °C. The results are presented in Figure 52 (n = 2).

gradient (n = 2)				
Protein	**Rep 1**	**Rep 2**	**Average**	**St. Dev.**
Cyt C	15.24		15.24	
Hemo	8.45	8.45	8.45	0.00
ProGRP	8.11	13.51	10.81	3.82
Myo	7.79	7.79	7.79	0.00
CaA				
HSA	2.46	2.46	2.46	0.00
Trans				
β-cat	3.97	3.97	3.97	0.00
IgG				
Fibr-alpha				
Fibr-beta		2.56	2.56	

Table 19: SQ % of protein Mix II (Table 13) digested for 5 min, 15 min, 30 min, 1 h and 2 h. The results are presented in Figure 54.

	5 min	**15 min**	**30 min**	**1 h**	**2 h**
Cyt C	33.33	10.48	39.05		
Hemo	8.45	6.34	6.34	31.69	
ProGRP	17.57	13.51	13.51	20.27	13.51
Myo	12.99	12.99	12.99	12.99	
CaA	8.08	8.46	8.46	8.08	
HSA		2.46	2.46	2.46	2.46
Transf		2.87		2.87	
β-cat	2.43		3.97	2.43	2.18
IgG					3.64
Fibr alpha					
Fibr beta				2.56	

Table 20: SQ % of three replicate injections of protein Mix II (Table 13) digested on-line in a 250 nL T/L immobilized OTERs. The data are presented in Figure 60.

OTER 1						
Protein	**SQ % Rep 1**	**SQ % Rep 2**	**SQ % Rep 3**	**SQ % Average**	**SQ % St. Dev.**	**SQ % RSD %**
Cyt C						
Hemo	38.03	38.03	38.03	38.03	0.00	0.00
ProGRP	20.27	25.68	33.11	26.35	5.26	19.97
Myo	88.96	83.12	85.06	85.71	2.43	2.83
CaA	79.23	79.23	79.23	79.23	0.00	0.00
HSA	18.23	11.17	22.66	17.35	4.73	27.27
Transf	40.83	41.83	41.69	41.45	0.44	1.07
β-cat						
IgG	7.88	7.88	7.88	7.88	0.00	0.00
Fibr alpha	44.72	43.09	42.28	43.36	1.01	2.34
Fibr beta	48.08	48.29	48.29	48.22	0.10	0.21
Fibr gamma	30.63	30.18	30.63	30.48	0.21	0.70
OTER 2						
Protein	**SQ % Rep 1**	**SQ % Rep 2**	**SQ % Rep 3**	**SQ % Average**	**SQ % St. Dev.**	**SQ % RSD %**
Cyt C	17.14		40.00	28.57	11.43	40.01
Hemo	31.69	38.03	38.03	35.92	2.99	8.32
ProGRP	22.97	15.54	25.68	21.40	4.29	20.03
Myo	79.43	83.12	88.96	83.84	3.92	4.68
CaA	90.38	95.00	79.23	88.20	6.62	7.50
HSA	13.79	11.66	26.77	17.41	6.68	38.36
Transf	39.11	39.97	38.48	39.19	0.61	1.56
β-cat		1.28		1.28	0.00	0.00
IgG	4.24	4.24	4.24	4.24	0.00	0.00
Fibr alpha	35.61	40.33	46.99	40.98	4.67	11.39
Fibr beta	32.26	44.02	33.55	36.61	5.27	14.38
Fibr gamma	22.75	28.15	25.00	25.30	2.21	8.75
OTER 3						
Protein	**SQ % Rep 1**	**SQ % Rep 2**	**SQ % Rep 3**	**SQ % Average**	**SQ % St. Dev.**	**SQ % RSD %**
Cyt C		12.38	17.14	14.76	2.38	16.12
Hemo	38.03	38.03	38.03	38.03	0.00	0.00
ProGRP	25.68	12.68	22.97	20.44	5.60	27.39
Myo	85.06	83.12	85.06	84.41	0.91	1.08
CaA	95.00	95.00	79.23	89.74	7.43	8.28
HSA	21.18	28.41	15.44	21.68	5.31	24.48
Transf	34.38	34.38	33.38	34.05	0.47	1.38
β-cat						
IgG	12.73	7.88	4.24	8.28	3.48	41.98
Fibr alpha	46.99	47.32	44.88	46.40	1.08	2.33
Fibr beta	41.67	49.79	43.80	45.09	3.44	7.62
Fibr gamma	31.08	36.49	30.41	32.66	2.72	8.33

Table 21: SQ % of protein Mix II (Table 13) digested in-solution with Lys-C, trypsin and T/L. Data presented in Figure 82.

Lys-C	HSA	Transf	Fibr-alpha	CaA	Myo	Fibr-beta	Hemo	Fibr-gamma	IgG	Cyt C	β-cat
Lys-C_1	7.88	2.01	11.38	10.38	38.96	14.74	46.48	15.32	16.06	N/A	0.00
Lys-C_2	6.57	2.15	16.10	24.62	17.53	14.74	34.51	11.49	10.30	N/A	0.00
Lys-C_3	8.70	1.86	9.11	23.85	39.61	21.37	34.51	20.27	22.12	N/A	0.00
Average	7.72	2.01	12.20	19.62	32.03	16.95	38.50	15.69	16.16	N/A	0.00
St. Dev.	1.07	0.15	3.57	8.01	12.56	3.83	6.91	4.40	5.91	N/A	0.00
Trypsin	**HSA**	**Transf**	**Fibr-alpha**	**CaA**	**Myo**	**Fibr-beta**	**Hemo**	**Fibr-gamma**	**IgG**	**Cyt C**	**β-cat**
Trypsin_1	15.44	12.61	22.11	41.92	48.05	15.6	38.03	14.19	13.33	18.1	0
Trypsin_2	16.26	5.87	10.73	35.38	59.74	7.05	34.51	8.33	13.33	18.1	0
Trypsin_3	50.32	53.72	48.94	75.77	85.06	45.3	34.51	31.98	44.24	36.19	4.1
Average	27.34	24.07	27.26	51.02	64.28	22.65	35.68	18.17	23.63	24.13	1.37
St. Dev.	19.91	25.90	19.62	21.68	18.92	20.08	2.03	12.32	17.85	10.44	2.37
T/L	**HSA**	**Transf**	**Fibr-alpha**	**CaA**	**Myo**	**Fibr-beta**	**Hemo**	**Fibr-gamma**	**IgG**	**Cyt C**	**β-cat**
T/L_1	28.90	24.64	33.17	56.92	59.09	31.20	49.30	34.46	20.91	38.10	12.80
T/L_2	31.53	27.22	32.85	68.46	59.74	37.39	49.30	32.66	17.88	54.29	14.60
T/L_3	30.21	25.21	33.50	68.46	59.74	36.32	49.30	39.41	17.88	33.33	11.14
Average	30.21	25.69	33.17	64.61	59.52	34.97	49.30	35.51	18.89	41.91	12.85
St. Dev.	1.32	1.36	0.33	6.66	0.38	3.31	0.00	3.50	1.75	10.99	1.73

9.2 Additional figures

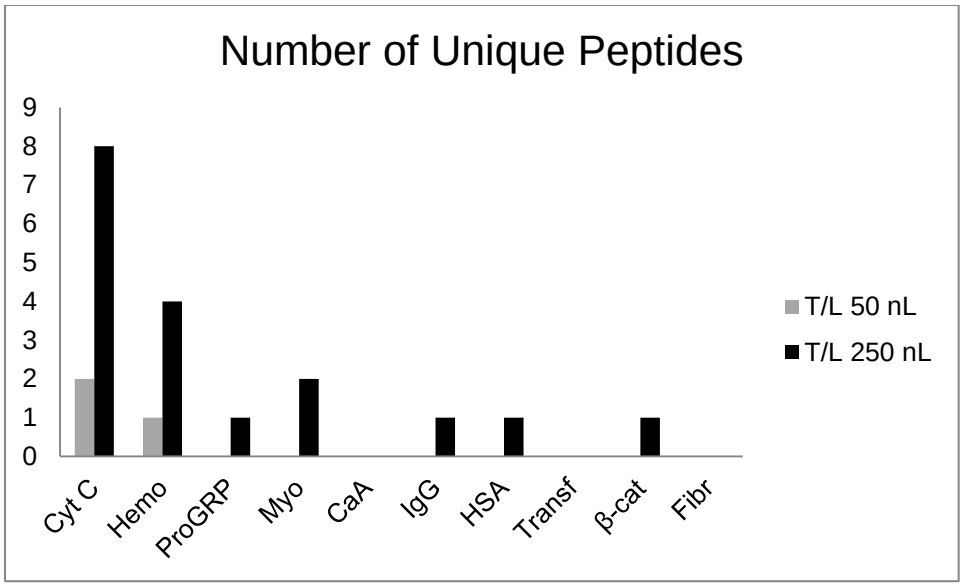

Figure 65: Number of unique peptides identified from a 50 nL OTER and 250 nL OTER when both were immobilized with T/L. Protein Mix II (Table 13) was injected into both OTERs. This is a supplementary to Figure 48.

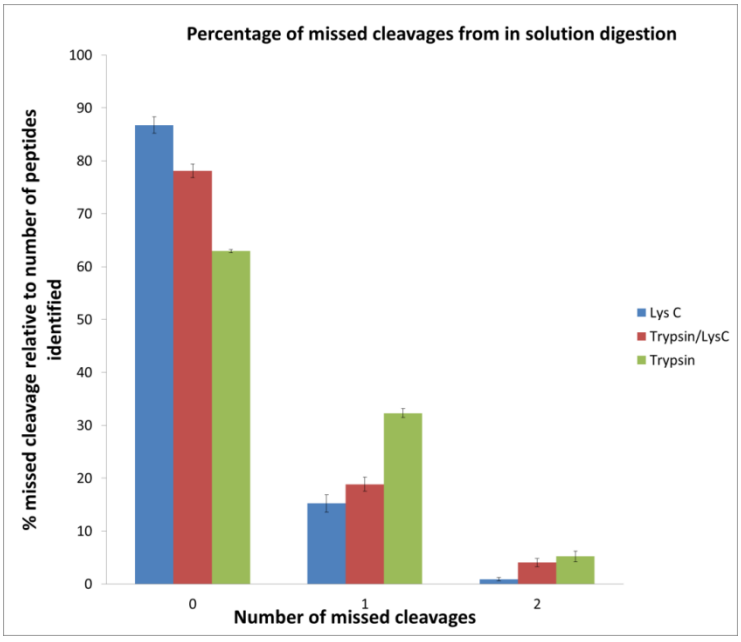

Figure 66: Percentage of missed cleavages from in-solution digestion of protein Mix I (Table 12) with Lys-C, T/L and trypsin (n = 3). The figure is a supplementary to Figure 82.

9.3 Posters

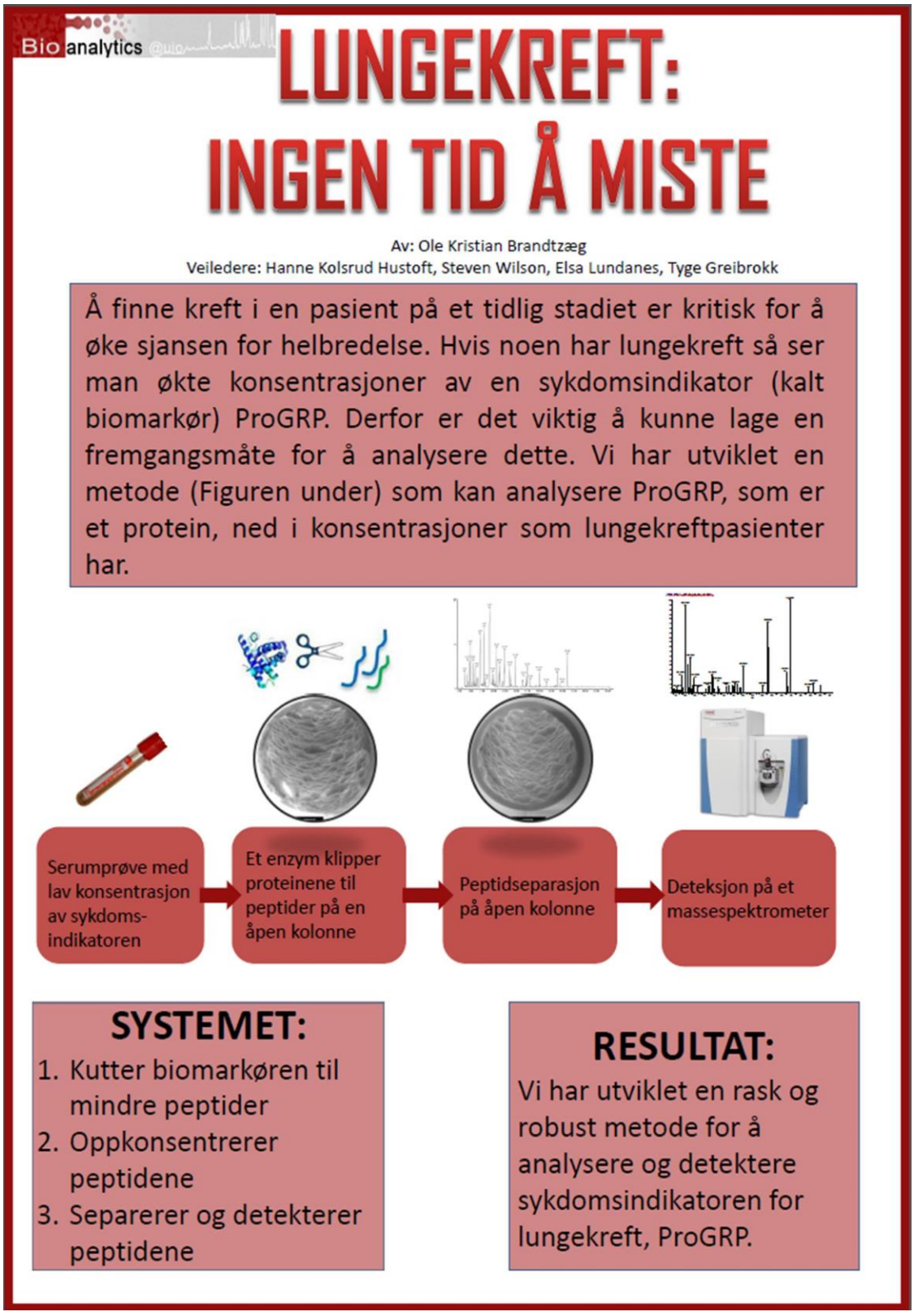

Figure 67: Poster presented at the Kjemi Grand Prix competition 2013 at the Department of Chemistry (UiO).

Bio analytics @uio

Development of a nanoLC-MS platform for the detection of low abundant proteins and biomarkers

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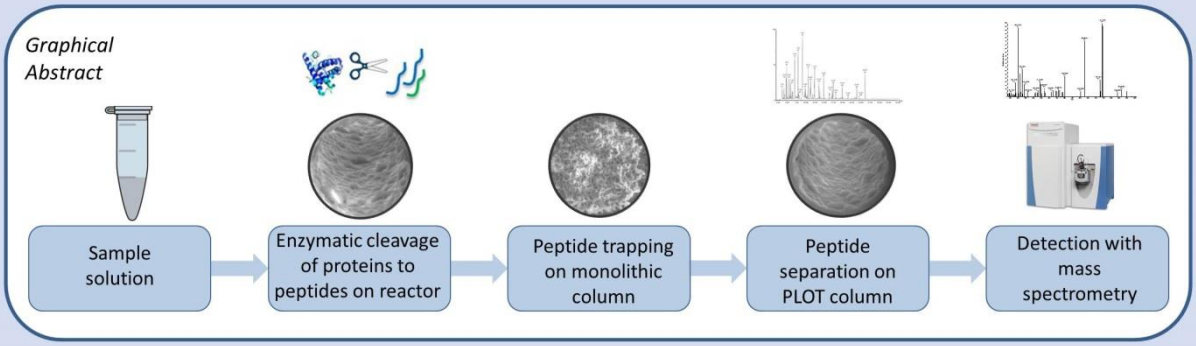

Introduction

Miniaturization of proteomic platforms is becoming more desirable in areas such as cancer research or diagnostics due to limitation of sample sizes and the low abundance of biomarkers [1]. To obtain high recovery and less contamination for limited sample volumes, a selective and sensitive on-line sample preparation is necessary [2].

In the present study, an automatable system for on-line enzymatic cleavage of proteins followed by peptide enrichment and subsequent separation has been developed. All columns were produced in-house.

Experimental

A novel open tubular trypsin reactor (20 µm ID, 150 mm) based on a polymer consisting of 2-hydroxyethyl methacrylate and vinyl azlactone was integrated in an on-line switching system together with a polystyrene divinylbenzene (PS-DVB) monolithic column (50 µm ID, 45 mm) for desalting and peptide enrichment, and a PS-DVB porous layer open tubular (PLOT) column (10 µm ID, 1 m) for reversed phase peptide separation with subsequent mass spectrometric detection on a Q Exactive Orbitrap (Figure 1).

Results

The trypsin reactor could cleave proteins in <30 min, making it suitable in an on-line chromatographic system. High resolution peptide separation resulted in narrow chromatographic peaks of 3–5 seconds (Figure 2). 300 attomole of the small cell lung cancer biomarker ProGRP was detected in the platform. The novel trypsin reactors provided repeatable performance and were compatible with long-term storage.

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References

[1] S. Surinova, R. Schiess, R. Hüttenhain, F. Cerciello, B. Wollscheid, R. Aebersold, Journal of Proteome Research 10 (2010) 5.
[2] J.-H. Kim, D. Inerowicz, V. Hedrick, F. Regnier, Analytical Chemistry 85 (2013) 8039.

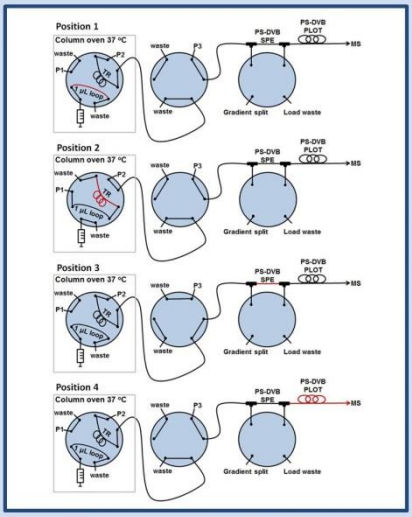

Figure 1: The workflow of the nanoLC-MS platform. Position 1: Sample injection. Position 2: Loading and subsequent digestion on trypsin reactor. Position 3: Peptide trapping. Position 4: PLOT LC separation with 40 nL/min flow. Washing and reconditioning of trypsin reactor. The red line represents the sample.

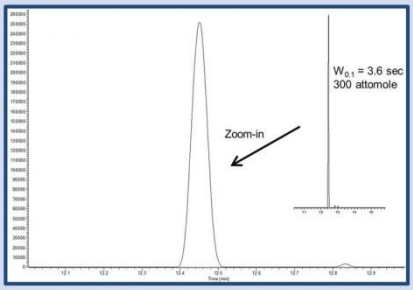

Figure 2: Extracted ion chromatogram of the signature peptide NLLGLIEAK (485.8^{2+}). $W_{0.1}$ = 3.6 sec (485.8^{2+}) from 300 attomole on-line digested ProGRP separated and detected in the novel nanoproteomic platform with PLOT peptide separation.

Figure 68: Poster presented at the 21st Kromatografisymposiet in Sandefjord, Norway.

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Integrated enzyme reactor and high resolving chromatography in "sub-chip" dimensions for sensitive protein mass spectrometry

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Reliable, sensitive and automatable analytical methodology is of great value in e.g. cancer diagnostics. In this context, an on-line system for enzymatic cleavage of proteins, subsequent peptide separation by liquid chromatography (LC) with mass spectrometric detection has been developed using "sub-chip" columns (10–20 μm inner diameter, ID). The system could detect attomole amounts of isolated cancer biomarker progastrin-releasing peptide (ProGRP), in a more automatable fashion compared to previous methods. The workflow combines protein digestion using an 20 μm ID immobilized trypsin reactor with a polymeric layer of 2-hydroxyethyl methacrylate-vinyl azlactone (HEMA-VDM), desalting on a polystyrene-divinylbenzene (PS-DVB) monolithic trap column, and subsequent separation of resulting peptides on a 10 μm ID (PS-DVB) porous layer open tubular (PLOT) column. The high resolution of the PLOT columns was maintained in the on-line system, resulting in narrow chromatographic peaks of 3–5 seconds. The trypsin reactors provided repeatable performance and were compatible with long-term storage.

The determination of biomolecules in areas like cancer research or diagnostics are driven towards liquid chromatography (LC) combined with mass spectrometry (MS) due to their low abundance, need for selectivity and the limitation of sample sizes from various biospecimens[1]. Also, on-line sample preparation steps are currently being developed for less contamination and high recovery of small "precious" samples[2]. Regarding selectivity, the combination of LC and MS is considered a benchmark technology, as it can distinguish e.g. isoforms and subtle post-translational modifications. To improve sensitivity, miniaturized columns are used as the column inner diameter (ID) is inversely proportional with signal intensity when using a concentration sensitive detector like the electrospray MS[3]. The commercial nanoLC columns/chips systems (≈50–75 μm column ID) coupled to nanospray MS are robust and commonly implemented in routine analysis[4,5].

To push sensitivity and thus detection limits even further, open tubular nanoLC columns with a stationary phase attached to the walls are arguably well suited, as the inner diameter of these columns is typically "sub-chip", e.g. 10–20 μm. Karger and co-workers first investigated the use of such narrow ID porous layer open tubular (PLOT) columns for LC separations of peptides[6–8]. Rogeberg *et al.* later showed the separation of intact proteins on PS-DVB PLOT columns and found these columns to be highly efficient and giving low carry-over[9]. Such PLOT columns have lately been used for the sensitive identification of proteins from 1,000–10,000 cancer cells[10,11].

A recent trend in bioanalysis is the development of proteomic platforms with on-line sample preparation[2,12], with selective sample enrichment[13], or for chemical reactions such as trypsin digestion[2] to cleave proteins into easier detectable/identifiable peptides. Immobilized enzyme reactors (IMERs) have several advantages compared to in-solution based approaches, such as larger enzyme to substrate ratio, higher digestion efficiency, the possibility to be reused and to be used in on-line LC-MS platforms[14]. Numerous immobilized trypsin reactors have been developed with a handful commercially available, typically as particle packed or monolithic columns[15–17]. Recently, the combination of a porous layer inside a capillary with the enzyme bound to the porous surface was reported[18,19].

Figure 69: Front page of published paper by Hustoft et al. in Scientific Reports by Nature [27]. Contributions of the author are stated in the paper.

9.4 Know-hows in column production

Tip 1 for column preparation

The capillary is often coiled for practical reasons.

Tip 2 for column preparation

The column preparation steps should be executed subsequently. The pre-treatment with NaOH, for activating the silanol groups, should be done overnight. When arriving the next morning the washing and drying of the column takes place while preparing the silanization solution. After the washing and drying steps are complete, the silanization solution is filled into the capillary. The capillary is the placed in the oven for 6 h. While the washing and drying steps after the silanization step takes place, the polymerization solution is prepared and filled into the capillary when the washing and drying steps have completed. The polymerization is then initiated right away before leaving the lab. The next morning the washing and drying steps after the polymerization are executed.

Tip 3 for column preparation

If the capillary is not flushed right after polymerization, the inlet and outlet could dry in contact with air causing clogging of the capillary. Cut a couple of cm of each side of the column, and it will be easier to flush them.

Tip 4 for column preparation

If AIBN is not properly solved in the polymerization mixture before placed in the oven, then AIBN sediment in the column and an uneven polymerization will take place. Trouble flushing the capillary afterwards could be encountered. Make sure to sonicate the polymerization solution, and make sure that there are no sediments swirling around in the solution before filling the capillary.

Tip 5 for column preparation

When the polymerization reaction takes 10 h, as in the case of OTERs, the reaction should be initiated immediately in order to avoid sedimentation of AIBN. The oven can be programmed to room temperature for the remaining h until flushing of the capillary will be performed the next morning.

Tip 6 for column preparation

It is important to enclose the sides of the capillary before turning off the N_2 pressure inside the bomb. Let the N_2 run for another 3 min before letting the pressure out of the bomb. Then quickly pull the capillary out of the bomb, and enclose the ends with a GC septa.

Tip 7 for column preparation

AIBN should be the last reagent added to the polymerization solution. If added first, then possible polymerization can take place before forced into the capillary by the pressure bomb. Hence, add the monomers and porogens first, and then the AIBN before placing the vial with the polymerization solution in an ultrasonic bath for 5 min.

10 Appendix 2

10.1 Side project: Finding a recipe for monolithic column with HEMA and VDM as monomers

Different combinations of monomers and porogens can alter the structure of the polymer. A monolithic column could give increased surface area, and hence more immobilized enzyme onto the polymer. The following shows how we found a recipe for a monolithic column with HEMA and VDM as monomers that can be immobilized with enzymes. A study to compare OTERs with monolithic columns immobilized with enzymes should be performed in the future. The following experiments were not a part of the main thesis, but were performed due to curiosity around monolithic columns polymerized with HEMA and VDM as monomers.

10.2 Introduction

Protein analysis, bottom-up, consists of protein digestion, separation of resulting peptides and mass spectrometric detection [111]. Digestion on a monolithic support gives high localized concentration of the enzyme. In addition, autodigestion is precluded and the digestion is significantly accelerated [105]. There have been several published papers on monolithic support for enzymatic digestion of proteins [105,112,113], and they show promising results.

10.3 Experimental

See main thesis for chemicals, solvents and experimental procedure of the production of columns (section 5.2).

10.3.1 Procedure for making HEMA-VDM monolith

The pre-treatment and silanization process were the same as used in the main thesis.

For the polymerization solution, the reagents were measured out using a micropipette and a scale. The polymerization solution was filled into the 50 µm ID capillary from Polymicro Technologies. The polymerization solution was altered using different amounts of HEMA, VDM, EDMA, 1-decanol and AIBN. The polymerization temperature was kept the same as

for the OT HEMA-VDM polymerized columns, namely 65 °C for 5 h and 80 °C for 5 h. The different combinations of monomers, porogens and amount of initiator that were tested are listed in Table 22.

Table 22: Ratios of monomers, porogens and initiator used in order to make 50 µm ID HEMA-VDM monolithic columns.

	% HEMA	% VDM	% EDMA	% 1-decanol	% AIBN	ID of column (µm)
Column MA	11.5	2.9	0.0	85.6	1.01	50
Column MB	10.9	2.7	5.0	81.4	0.74	50
Column MC	10.9	2.7	5.0	81.4	0.08	20
Column MD	10.9	2.7	5.0	81.4	0.74	20
Column ME	20.1	5.0	0.0	74.9	0.05	20

10.3.2 Column Characterization

For column characterization, the FEI Quanta 200 FEG-ESEM was used. The low vacuum mode was used with a LFD detector. The parameters for magnitude, working distance, spot size and voltage were altered in order to obtain the best resolution.

10.4 Results and Discussion

The following results are qualitative, and the columns are characterized by the use of SEM.

10.4.1 Characterization of HEMA-VDM monolithic columns

Column MA-ME, in Figure 70, shows the resulting monolith for the corresponding recipes in Table 22. Increasing the amount of AIBN more than a tenfold with respect to the initial recipe used for OT (see main thesis) a monolithic structure is observed. Usually a crosslinker, such as EDMA, is needed in order to make a polymer network. A crosslinker has been added to the original recipe for column MB. Columns MC to ME were less successful. Whether these monolithic columns, polymerized with HEMA and VDM, will be able to digest proteins after immobilization with enzymes need to be investigated in the future.

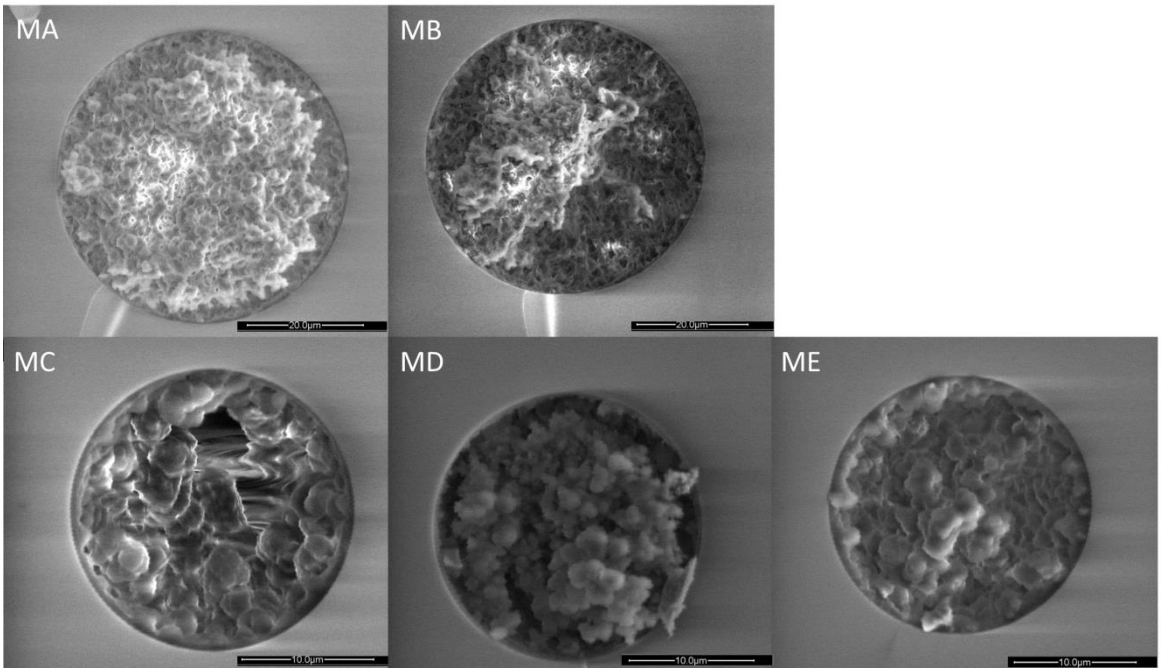

Figure 70: SEM images of 50µm ID HEMA-VDM monolithic column production attempts.

10.5 Conclusion

A recipe (column MA) for a monolithic column polymerized with HEMA and VDM as monomers was successfully developed. These columns should be immobilized with enzymes in the future and the digesting efficiency should be compared with OTERs.

11 Appendix 3

11.1 Side project: Hybrid-columns

The following experiments were performed the first semester during the master program. To the authors knowledge such columns have not been prepared earlier.

11.2 Introduction

Packed columns need frits in order to keep the particles inside the capillary. If the capillary is cut at the middle, for example, then the packing material will be forced out of the column, and the LC-system will be clogged. Fritless packed columns can be cut at any point along the column without any packing material leaving the column [114].

Porous graphitized carbon (PGC), commercially known as Hypercarb (HC), are spherical and with a porosity of 75 %. The particles are synthesized by layers of hexagonally arranged carbon atoms. These atoms are linked by conjugated bonds. HC separation media has shown to be a good separation medium for polar compounds [115,116]. The polarizable surface of the graphite interact through a charge-induced interaction with the polar analyte. HC has shown to be an unique packing material that can use mobile phases of wide ranges of pH values to improve the separation of polar analytes [117].

Hybrid is a combination of two (or more) different elements. In the main thesis monolithic columns have been prepared (BuMa and PS-DVB). These monolithic columns do not need any frits because during polymerization they create a network of polymer that is attached to the capillary wall. By dissolving HC in the polymerization solution of a BuMa SPE column (see main thesis) recipe, a hypothesis was made that a polymer network would be created and keep the HC inside the capillary.

11.3 Experimental

See main thesis for the list of chemicals. HC (5 μm particles) was obtained from Thermo Fisher Scientific. 1 mg/mL ovotransferrin was in-solution digested and received from Helle Malerød. The LC-MS-system used for the testing of hybrid (type 2) was developed by Helle

Malerød. The MS was a Q Exactive Orbitrap from Thermo Fischer Scientific. Carbon tetrachloride was purchased from Sigma Aldrich.

A desired length of 50 µm ID capillary was cut. The pre-treatment and silanization procedure were as stated in the main thesis. A polymerization solution for BuMa monolithic SPE column was prepared (see main thesis). The capillary was filled with the hybrid solution by using a syringe, injection port and a union (Figure 71).

Figure 71: Setup used to fill the 50 µm capillaries with the hybrid polymerization and HC solution.

11.3.1 SEM procedure

A sample of the capillary (about 1 cm) was cut off, placed on a carbon tape, and brought to the FEI Quanta 200 FEG-ESEM (FEI, Hillsboro, OR, USA). The low vacuum mode was initiated while taking the SEM images and a LFD detector was used.

11.4 Results and discussion

Characterization of two types of hybrid columns consisting of HC and BuMa polymerization solution was performed.

11.4.1 Homogeneity of HC in the polymerization solution

To make a homogeneous column, the polymerization solution needs to be homogeneous. By making a slurry of 12 mg HC in 300 µL BuMa polymerization solution and sonication for 5 min, the mixture became homogeneous (Figure 72). However, within 5 min the HC sedimented at the bottom of the glass vial. Hence, the silanized capillary had to be filled with the hybrid (HC-BuMa) polymerization solution rapidly after sonication.

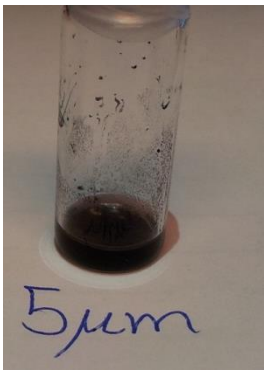

Figure 72: Homogeneous slurry of 12 mg HC mixed in 300 µL BuMa polymerization solution.

11.4.2 Preparation and characterization of hybrid column type 1

The procedure for preparing the hybrid column type 1 was as follows. 12 mg HC (5 µm in diameter) was dissolved in 300 µL of the BuMa polymerization solution. The capillaries were plugged in both ends with GC septum after filling before placed in the oven at 70 °C for 16 h. After polymerization the capillaries were flushed with ACN and dried with N_2 before being examined by microscope or SEM.

The hybrid column tolerated a back-pressure of 100 bar without any bleeding of polymer. It was further studied in a Motic microscope first in order to quickly establish the outcome of the polymerization and to compare with a capillary polymerized with BuMa (Figure 73). From this observation, it was thought that the polymer had been formed at the inside of the capillary, and the column also contained HC, because of the difference from the BuMa column. The BuMa and the hybrid columns were also compared by using the Olympus microscope (Figure 74). The HC column was also studied in the SEM (Figure 75), and it was observed that the column contained HC. However, the incorporation of HC was not satisfactory. Even though the hybrid column tolerated a back-pressure of 100 bar, it did not look like the HC were held tightly by the polymer as one of the particles was already on the outside during imaging. Taking a closer look with the SEM at one of the particles (Figure 76) it might seem like the polymer has covered the HC. The function of the HC being a good separation medium for polar compounds is therefore lost as the reversed phase separation media BuMa is covering the HC. Hence, these columns were not tested any further.

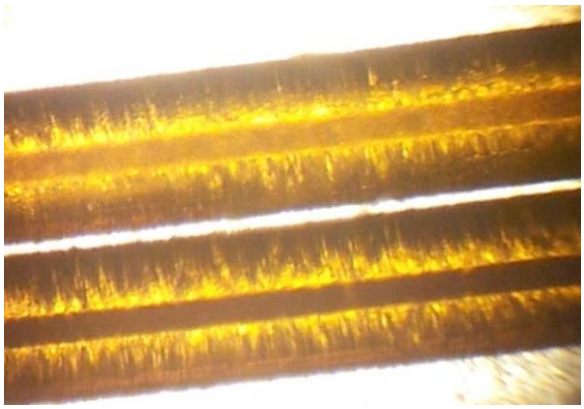

Figure 73: Microscope image of the hybrid column. Above: BuMa monolithic column. Below: hybrid column with HC and BuMa. 12 mg HC dissolved in 300 µL BuMa polymerization solution.

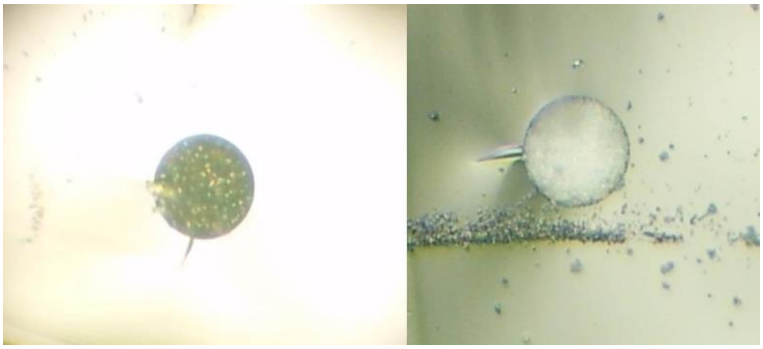

Figure 74: Image taken with the Olympus microscope. Left: Hybrid column. Right: BuMa column. 12 mg HC dissolved in 300 µL BuMa polymerization solution.

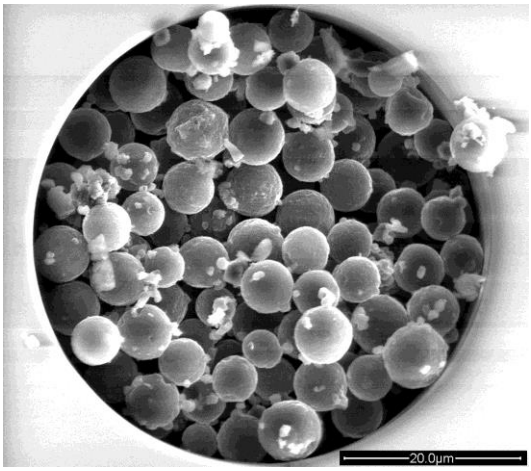

Figure 75: SEM image of hybrid column. 12 mg HC dissolved in 300 µL BuMa polymerization solution.

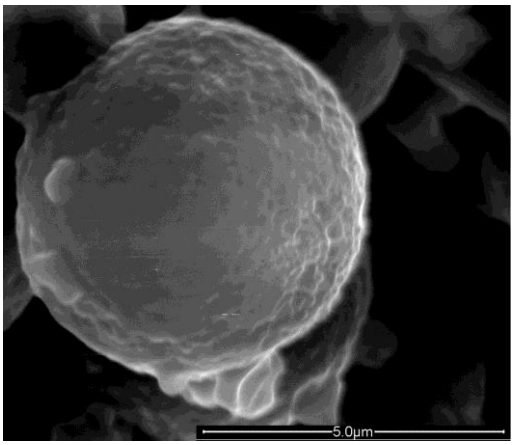

Figure 76: SEM image of HC particle inside the hybrid column.

11.4.3 Preparation and characterization of hybrid column type 2

Since the particles were covered by the polymer when dissolving the HC in BuMa polymerization solution, a new idea was tested, where the HC was packed towards a BuMa monolith.

The following procedure was used to prepare the hybrid column type 2. The BuMa polymerization solution was filled into the 50 µm capillary. Half of the capillary was placed inside the oven (pre-heated to 70 °C, see Figure 77), and hence only half of the capillary was polymerized when removing the capillary after 16 h. The remaining polymerization solution that had not been polymerized was back flushed out of the capillary by the use of a pressure bomb. The column was now ready for packing of 5 µm (in diameter) HC particles. An amount of 5 mg HC particles were mixed in 300 µL of carbon tetrachloride.

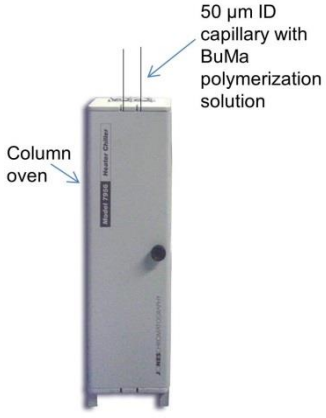

Figure 77: Illustration of how parts of a capillary can be polymerized.

Of the 50 µm capillary, 2 cm was polymerized with BuMa monolith, 2 cm was packed with HC and another 2 cm was left empty behind the HC (Figure 78).

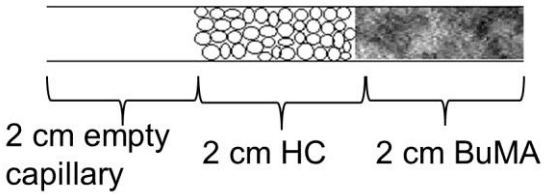

Figure 78: Illustration of the hybrid column (type 2).

To ensure that the BuMa monolith could hold the HC inside the column, increasing the back-pressure up to 350 bar was tested. No bleeding of the polymer of HC was observed.

The column was installed into a simple LC-MS-system (Figure 79). 50 nL of 1 mg/mL in-solution digested ovotransferrin was injected into the hybrid (type 2) column, and a SQ % of 51.63 was found (Figure 80). Hence, the hybrid column successfully trapped polar peptides before detection with MS.

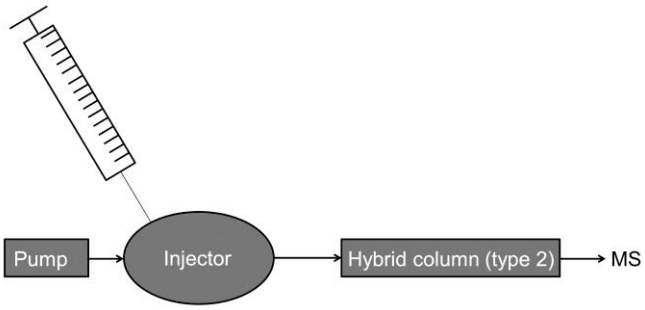

Figure 79: LC system used to evaluate if the hybrid (type 2) could retain polar peptides from ovotransferrin.

Further testing of the column was not performed as this was the point where the main thesis project started.

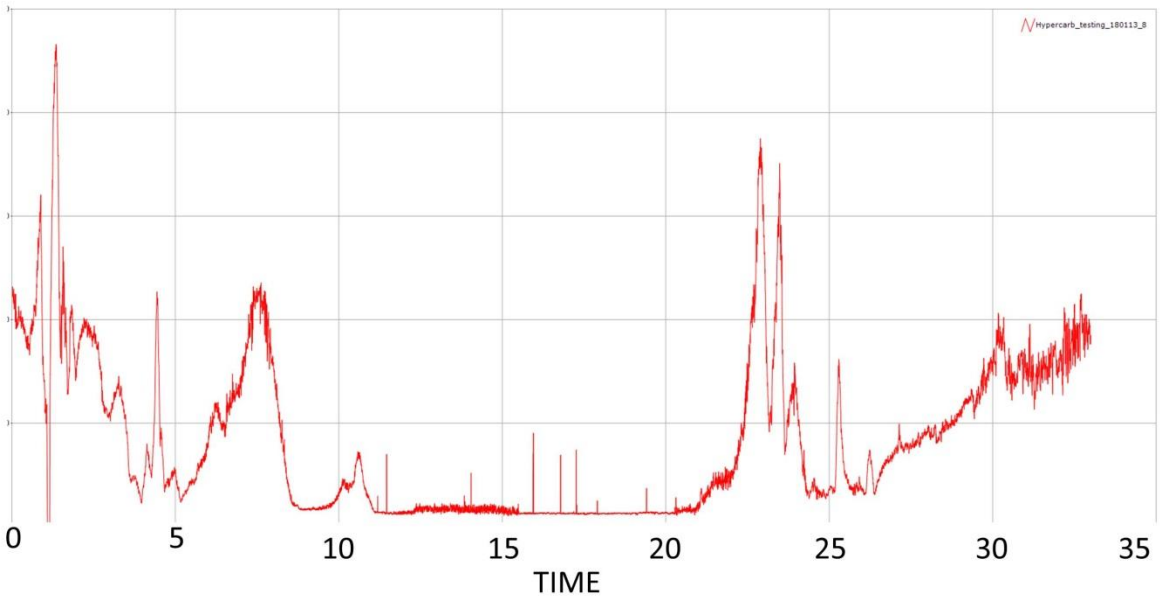

Figure 80: Full MS chromatogram of 50 nL of 1 mg/mL in-solution digested ovotransferrin.

12 Appendix 4

12.1 In-solution digestion of mix of 9 proteins

In-solution digestion of protein Mix II (Table 13 in Appendix) was performed in order to evaluate the efficiency of digestion in-solution overnight, and for use as reference when performing digestion on-line with the enzymatic reactor. ProGRP was not present in the protein mixture for in-solution digestion and was therefore omitted for comparisons. Cyt C was not present in the sample digested in-solution with Lys-C. The highlights in the chromatographic system used for this evaluation is shown in Figure 81.

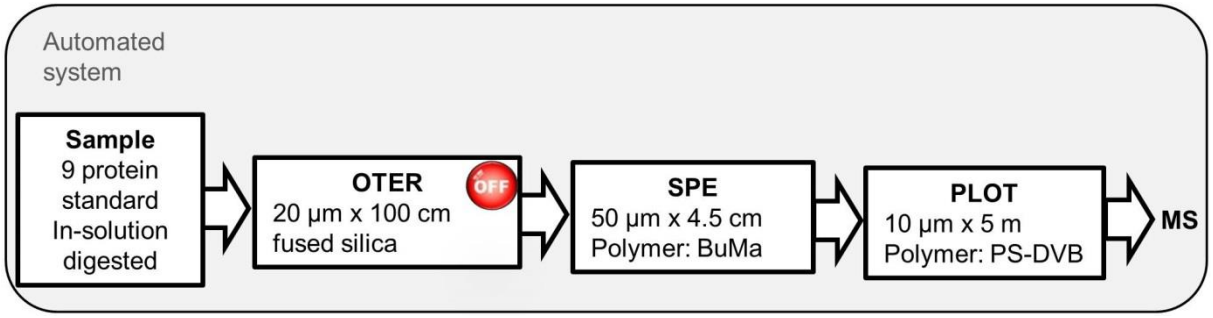

Figure 81: Highlights of the chromatographic system used for evaluation of the automated system.

The in-solution digestions were performed with trypsin, Lys-C and a mixture of trypsin and Lys-C. An amount of 5 µg of each protein was added to the mixture. Reduction and alkylation were performed, as described in the experimental section. The protocol stated in section 5.3.3.1 in experimental was performed for in-solution digestion with Lys-C and T/L. The protein mixture digestion by trypsin was performed by the protocol stated in section 5.3.3.2. Three replicates of each protein mixture were prepared and digested in-solution. The digested protein mixtures were analyzed in the automated system (without the OTER), which consisted of a 5 cm x 50 µm ID BuMa SPE column and a 5 m x 10 µm ID PS-DVB PLOT column.

From Figure 82, it is clear that Lys-C as digestion enzyme does not give as many peptides as trypsin and T/L. Trypsin and T/L could digest the same amount of proteins (data listed in Table 21 in Appendix).

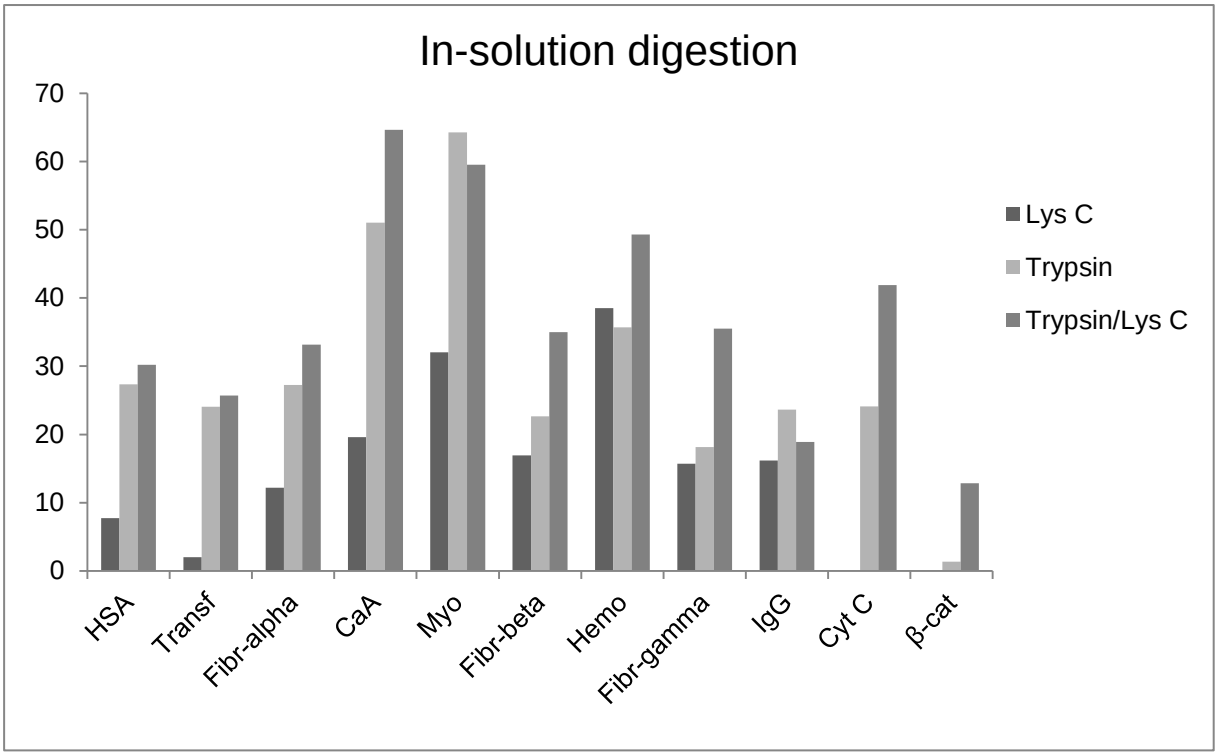

Figure 82: Average SQ % of three injections of a mixture with 9 proteins digested in-solution by trypsin, Lys-C and T/L. Cyt C was not present in the mixture digested in-solution with Lys-C. Precursor mass tolerance, which usually is set to 10 ppm, had to be increased to 100 ppm in the search parameters. Missed cleavages were set to 2. The peptides were considered identified if they were present when the result filters were set to medium peptide confidence level.

The SQ % of each protein was calculated by Sequest in Proteome Discoverer. The Orbitrap mass spectrometer had not been calibrated for a couple of days before the analysis was performed. Missed cleavages were expected to be lower for the protein mixture digested by Lys-C and T/L compared to the protein mixture digested by trypsin, as reported by Saveliev et al. [13]. This was also confirmed in this experiment. Figure 66 in Appendix shows the distributions of missed cleavages for the in-solution digestion, and Lys-C gave the lowest amount of missed cleavages, followed by T/L.