Optimization of in-house packing of nano liquid chromatography columns

How to pack low-cost nano liquid chromatography columns?

Henriette Sjånes Berg

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

Department of Chemistry
Faculty of Mathematics and Natural Sciences

UNIVERSITY OF OSLO
May 18th 2016
Optimization of in-house packing of nano liquid chromatography columns

How to pack low-cost nano liquid chromatography columns?

Henriette Sjånes Berg

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

Department of Chemistry
Faculty of Mathematics and Natural Sciences

UNIVERSITY OF OSLO

May 18th 2016
Optimization of in-house packing of nano liquid chromatography columns
A fast, simple and low-cost packing procedure for making nano liquid chromatography columns with excellent performance

Henriette Sjånes Berg

http://www.duo.uio.no/

Printed at Reprosentralen, Universitetet i Oslo
Abstract

Liquid chromatography-mass spectrometry (LC-MS) approaches for measurement of biomarkers are increasing in popularity due to the selectivity and sensitivity. To match the detection limits of the immunoassay approaches (such as enzyme-linked immunosorbent assay (ELISA) and Western Blot (WB)), LC columns with narrow inner diameter are preferable. The goal of this work was to establish a simple and low cost slurry packing procedure to obtain 50 µm inner diameter (ID) x 150 mm particle packed nanoLC columns with comparable performance to a 75 µm ID x 150 mm commercial nanoLC column. Slurry packing with 2.6 µm solid-core reversed phase particles was performed with an in-house made pressure bomb system (< 200 bar).

Column efficiency was measured as peak capacity (number of peaks separated in a gradient window, PC), calculated using peptides from tryptic digest of human serum albumin (HSA). To maintain sedimentation of particles, heat or magnetic stirring was needed. 80/20 % acetonitrile (ACN)/water as slurry solvent was found to give both the fastest packing and the most efficient columns measured as PC (89 ± 11 at 10 % of the peak height for columns packed with magnetic stirring). Nevertheless, the column performance was quite similar for all slurry solvents and packing approaches.

“Pico Frit” column housings from New Objective and two frit-making procedures (polymerization and sintering of silica particles) for standard column housings were compared, but none outperformed the others. The PC of the commercial column packed with the same material as the in-house packed columns, was approximately 30 % higher than the best performing in-house packed column (102 vs 136). However, the retention time repeatability between replicates was equal to the commercial column for most columns packed with 80/20 % ACN/water. In conclusion, this work presents a fast and low cost packing procedure of nanoLC columns with similar performance to commercial nanoLC columns.
Preface

The present work was mainly performed at the Department of Chemistry at the University of Oslo from August 2014 to May 2016.

I would like to use this opportunity to give a huge thanks to my supervisors; Professor Elsa Lundanes, Associate Professor Steven Ray Haakon Wilson, and PhD candidate Tore Vehus for the great support, excellent guidance and constructive feedback throughout my master’s degree. Thanks to Inge Mikalsen for additional technical support and troubleshooting. I greatly appreciate all the assistance!

I would also like to thank all students and other academic employees at the research group of Bioanalytical chemistry for creating a wonderful and including working environment, and to the Cell Signalling Unit at Rikshospitalet for including me the short time I was there. In this regard, I would give a special thanks to my office mates Kristina Erikstad Sæterdal and Tone Smetop for the cooperation with an application for the developed packing method described in the present thesis.

In addition, I would thank my family for the support throughout my degree, and a special thanks to my fiancé Kim A.O Engen for all the love, care and support, no matter what.

Oslo, Norway, May 2016

Henriette Sjånes Berg
# Table of content

1 Abbreviations and definitions ........................................................................................................... 1
  1.1 Abbreviations ................................................................................................................................. 1
  1.2 Definitions ....................................................................................................................................... 3
2 Introduction ......................................................................................................................................... 5
  2.1 Proteomics and early diagnostics .................................................................................................... 5
    2.1.1 Proteomic techniques .................................................................................................................. 6
    2.1.2 Top-down versus bottom-up proteomics ...................................................................................... 6
    2.1.3 Sample preparation in bottom-up proteomics ............................................................................. 7
  2.2 Liquid chromatography – mass spectrometry .................................................................................. 11
    2.2.1 Mass spectrometry ..................................................................................................................... 11
    2.2.2 Electrospray ionization mass spectrometry ............................................................................... 13
    2.2.3 Reversed phase LC-MS .............................................................................................................. 17
  2.3 Chromatographic performance ....................................................................................................... 21
    2.3.1 Column band broadening .......................................................................................................... 21
    2.3.2 Extra column band broadening .................................................................................................. 23
  2.4 Miniaturized liquid chromatography .............................................................................................. 25
    2.4.1 Decreasing column inner diameter (nanoLC) ............................................................................ 25
    2.4.2 Frits ............................................................................................................................................ 27
    2.4.3 Large volume injections ............................................................................................................. 28
  2.5 Column packing technology ........................................................................................................... 29
  2.6 Aim of study ..................................................................................................................................... 36
3 Experimental .......................................................................................................................................... 37
  3.1 Chemicals ........................................................................................................................................ 37
  3.2 Materials and equipment .................................................................................................................. 38
  3.3 Column packing set-up ..................................................................................................................... 40
  3.4 LC-MS instrumentation .................................................................................................................... 41
    3.4.1 MS detection ............................................................................................................................... 41
    3.4.2 Computer software ...................................................................................................................... 42
    3.4.3 Pumps/autosamplers .................................................................................................................. 42
    3.4.4 Connections ............................................................................................................................... 42
    3.4.5 LC-system settings ..................................................................................................................... 43
6.4.4 Comparison of neat slurry solvents and binary solvents................................. 95
6.4.5 Investigation of C\textsubscript{30} vs C\textsubscript{18} in nanoLC format ........................................ 97
6.5 Raw data and calculations (tables) ........................................................................ 101
  6.5.1 Investigation of conventional C\textsubscript{18} and C\textsubscript{30}........................................ 101
  6.5.2 In-house packing of nanoLC columns ................................................................. 103
6.6 Supplementary chromatograms ............................................................................ 108
6.7 Standard operation procedure (SOP) for packing of nanoLC columns................. 109
6.8 Publications ........................................................................................................... 111
# Abbreviations and definitions

## 1.1 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>A</td>
<td>Constant related to eddy dispersion</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AGC</td>
<td>Automatic gain control</td>
</tr>
<tr>
<td>A&lt;sub&gt;s&lt;/sub&gt;</td>
<td>Asymmetry factor</td>
</tr>
<tr>
<td>B</td>
<td>Constant related to longitudinal diffusion</td>
</tr>
<tr>
<td>C</td>
<td>Constant related to resistance to mass transfer</td>
</tr>
<tr>
<td>c&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Analyte concentration in the sample</td>
</tr>
<tr>
<td>c&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Analyte concentration at peak maximum</td>
</tr>
<tr>
<td>D</td>
<td>Radial dispersion (dilution)</td>
</tr>
<tr>
<td>d&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Particle density</td>
</tr>
<tr>
<td>d&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Solvent density</td>
</tr>
<tr>
<td>d&lt;sub&gt;c&lt;/sub&gt;</td>
<td>Column inner diameter</td>
</tr>
<tr>
<td>ddMSMS</td>
<td>Data-dependent tandem mass spectrometry</td>
</tr>
<tr>
<td>D&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Diffusion constant of a solute in a mobile phase</td>
</tr>
<tr>
<td>d&lt;sub&gt;p&lt;/sub&gt;</td>
<td>Particle diameter</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EIC</td>
<td>Extracted ion chromatogram</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization, ionspray</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>f</td>
<td>Sensitivity factor</td>
</tr>
<tr>
<td>f(k)</td>
<td>Function dependent on the retention factor</td>
</tr>
<tr>
<td>Fwhm</td>
<td>Full width half maximum (band width at 50% of the peak height)</td>
</tr>
<tr>
<td>g</td>
<td>Gravitational constant</td>
</tr>
<tr>
<td>H</td>
<td>Plate height</td>
</tr>
<tr>
<td>HCD</td>
<td>Higher energy collisional dissociation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>IAM</td>
<td>Iodoacetamide</td>
</tr>
<tr>
<td>ID</td>
<td>Inner diameter</td>
</tr>
<tr>
<td>k</td>
<td>Retention factor</td>
</tr>
<tr>
<td>Kasil</td>
<td>Potassium silicates</td>
</tr>
<tr>
<td>L</td>
<td>Length of a column</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>Leu-Enk</td>
<td>Leucine enkephalin</td>
</tr>
<tr>
<td>LHRH</td>
<td>Luteinizing hormone releasing hormone</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass to charge ratio</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>Met-Enk</td>
<td>Methionine enkephalin</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
</tr>
<tr>
<td>n</td>
<td>Replicate(s)</td>
</tr>
<tr>
<td>N</td>
<td>Plate number</td>
</tr>
<tr>
<td>nanoESI</td>
<td>Nanospray (electrospray ionization for low flow rates)</td>
</tr>
<tr>
<td>nanoLC</td>
<td>Nano liquid chromatography</td>
</tr>
<tr>
<td>OD</td>
<td>Outer diameter</td>
</tr>
<tr>
<td>P</td>
<td>Pressure</td>
</tr>
<tr>
<td>PC</td>
<td>Peak capacity</td>
</tr>
<tr>
<td>PEEK</td>
<td>Polyetheretherketone</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>r_c</td>
<td>Column radius</td>
</tr>
<tr>
<td>RF</td>
<td>Radio frequency</td>
</tr>
<tr>
<td>r_p</td>
<td>Particle radius</td>
</tr>
<tr>
<td>RPLC</td>
<td>Reversed phase liquid chromatography</td>
</tr>
<tr>
<td>RSD%</td>
<td>Relative standard deviation in percent</td>
</tr>
<tr>
<td>S_N2</td>
<td>One-step nucleophilic substitution reaction</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>SPE-LC</td>
<td>On-line solid phase extraction liquid chromatography</td>
</tr>
</tbody>
</table>
STD  Absolute standard deviation
\( t_a \)  Retention time of the first eluting compound in a gradient window
TCEP  Tributylphosphine
TFA  Trifluoroacetic acid
TIC  Total ion chromatogram
\( t_R \)  Retention time
\( t_z \)  Retention time of the last eluting compound in a gradient window
\( u \)  Flow rate
UPLC  Ultra performance/pressure liquid chromatography (product of Waters)
\( v \)  Sedimentation velocity
\( V_{\text{max}} \)  Maximum injection volume without extra band broadening
\( w_{0.5} \)  Peak width at 50 % of the peak height
\( w_{\text{av}} \)  Average peak width (10 % or 50 % of the peak height)
\( \gamma \)  Constant
\( \varepsilon_t \)  Particle porosity
\( \eta \)  Viscosity
\( \kappa \)  Constant
\( \lambda \)  Constant dependent on particle shape
\( \sigma^2_x \)  Variance of a parameter “x”

### 1.2 Definitions

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active site</td>
<td>Site on an protein/peptide where a substrate can bind</td>
</tr>
<tr>
<td>Agglomeration</td>
<td>Reversible clustering of particles</td>
</tr>
<tr>
<td>Aggregation</td>
<td>Irreversible clustering of particles</td>
</tr>
<tr>
<td>Automatic gain control</td>
<td>Maximum number of ions collected for scan or fragmentation in the MS</td>
</tr>
<tr>
<td>Base peaks</td>
<td>The peaks with the highest intensities in a mass spectrum</td>
</tr>
<tr>
<td>Biomarker</td>
<td>Biomolecules used for monitoring of diseases</td>
</tr>
<tr>
<td>Cross-link</td>
<td>A bond/binding of polymer chains</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>----------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>ddMSMS</td>
<td>Fragmentation of base peaks</td>
</tr>
<tr>
<td>Denaturation</td>
<td>Process where proteins loses the native quaternary, tertiary and secondary structure.</td>
</tr>
<tr>
<td>Detection limit</td>
<td>Lowest amount of analyte detectable</td>
</tr>
<tr>
<td>Dynamic range</td>
<td>Difference in concentration between the most abundant protein and the least abundant protein</td>
</tr>
<tr>
<td>Endoprotease</td>
<td>Protease which cleaves a protein inside the polypeptide chain</td>
</tr>
<tr>
<td>Enzyme</td>
<td>A protein catalyzing biochemical reactions</td>
</tr>
<tr>
<td>Eukaryotes</td>
<td>Cells with organelles enclosed with a membrane (e.g. human cells)</td>
</tr>
<tr>
<td>Focusing time</td>
<td>The elution time with low organic content before gradient start</td>
</tr>
<tr>
<td>Gradient elution</td>
<td>Elution with changing mobile phase compositions during the analysis</td>
</tr>
<tr>
<td>in silico</td>
<td>Performed by a computer or computer simulations</td>
</tr>
<tr>
<td>Injection time</td>
<td>The maximum time the MS uses to collect ions before scan or fragmentation.</td>
</tr>
<tr>
<td>Isocratic elution</td>
<td>Elution with constant mobile phase composition</td>
</tr>
<tr>
<td>Loading capacity</td>
<td>Maximum sample loading amount on a column without extra band broadening</td>
</tr>
<tr>
<td>Nucleophilic species</td>
<td>Chemical species which form a chemical bond to an electrophilic species by donating an electron pair</td>
</tr>
<tr>
<td>Permeability</td>
<td>Lower backpressure at the same flow rate</td>
</tr>
<tr>
<td>Precursor ions</td>
<td>Intact ions from the ionization source</td>
</tr>
<tr>
<td>Protease</td>
<td>Protein cleaving enzyme</td>
</tr>
<tr>
<td>Proteolytic active agents</td>
<td>Protein cleaving agents with a high degree of cleavages.</td>
</tr>
<tr>
<td>Proteomics</td>
<td>Large scale studies of the protein content in cells/tissue etc.</td>
</tr>
<tr>
<td>Sedimentation time</td>
<td>The time before all particles in a slurry have settled</td>
</tr>
<tr>
<td>Signature peptide</td>
<td>Peptide specific for a given protein</td>
</tr>
<tr>
<td>Size segregation</td>
<td>Radially segregation of particles with different diameter</td>
</tr>
</tbody>
</table>
# 2 Introduction

## 2.1 Proteomics and early diagnostics

Early detection of biomarkers for diseases, such as cancer, is one of the most challenging and important goals in clinical medicine [1-3]. To succeed, optimization and advancements regarding efficiency, simplicity and cost in all aspect of the process, from sampling to interpretation of results, are important.

The study of gene expression can be divided into two main approaches; the use of nucleic acid-based technology, and the protein-based technology [4]. The first approach deals with deoxyribonucleic acid (DNA) and messenger ribonucleic acid (mRNA), which contain the whole set of genes that can be expressed as proteins. On the other hand, the protein-based technology, or proteomics, attempts to monitor biological processes, cell functions and signaling pathways [5]. One definition of proteomics is given by Rodriguez-Suarez et al.: “the study of the proteome of a cell or tissue at a given time, where the proteome is defined as the total protein complement” [6]. By measuring proteins or peptides, which are smaller parts of a protein, gene and cellular function can be determined [7].

In general, all proteomic techniques suffer from several challenges, primary due to the matrix from which the proteins are extracted. Typically, proteins are extracted from cells, tissue, single organelles, or serum [2, 8-10]. All these matrices have a high degree of complexity as well as a high dynamic range of protein concentration. The latter is one of the main challenges in analytical chemistry today [11]. Some biomarkers (compounds related to diseases) are found in very low concentrations in matrices with typically small sample volumes, and thus require high sensitivity methods for determination. Also robustness, price and time consumption are common challenges for the instrumentation in proteomics [2].
2.1.1 Proteomic techniques

For proteomic experiments, immunoassay methods such as ELISA and WB have frequently been used [12-14]. These methods are using antibodies specific (i.e. intended for) for an analyte protein/peptide for identifications or quantifications, and are commonly used in proteomics due to their sensitivity and ease of use. However, the development of antibodies with high selectivity (for reliable protein identifications or quantifications) has proven to be a major challenge [15, 16]. Hence, the excellent selectivity, increased sensitivity and dynamic range of recent MS instrumentation have increased the popularity of MS based methods in proteomics [16, 17].

2.1.2 Top-down versus bottom-up proteomics

In recent years, top-down and bottom-up approaches have been developed for immunoassays, gel-based separation techniques in addition to LC-MS [2, 10, 18-20]. In top-down approaches, intact/whole proteins are measured by LC-MS, while in bottom-up approaches, proteins are cleaved to peptides (i.e. 2-100 amino acids in sequence) with (usually) a protease (protein cleaving enzyme) before determination by LC-MS (Figure 1).

Commonly, bottom-up approaches have been preferred because of earlier MS instrumentation limitations (traditionally mass ranges have been limited to approximately 20 amino acids [17]). However, top-down methods have increased in popularity due to time saving in the sample preparation and more structural information of the proteins. The main reason for choosing a bottom-up approach is the better solubility of peptides in the mobile phases commonly used in LC-MS. The solubility of intact proteins is more restricted. For example, hydrophobic membrane proteins and hydrophilic body fluid proteins would not be soluble in the same solvent. On the other hand, bottom-up approaches only give partially information about the protein, but usually enough to identify and quantify a protein. [17, 21]
2.1.3 Sample preparation in bottom-up proteomics

The sample preparation in bottom-up proteomics is typically performed “in-gel” or “in-solution”. For the “in-solution” approach used in the present study, the proteins are extracted from the matrix and dissolved in an appropriate buffer for digestion. The digestion itself consist of several steps; reduction and alkylation under denaturing conditions to maximize the number of possible cleavages, the proteolytic digestion, and often a desalting step to remove unreacted reagents, possible interferences and particles that can cause clogging of the analytical column [17, 22, 23]. The sample preparation should consist of as few steps as possible to reduce the possibility of contamination. All surfaces (pipets, tubes, etc.) have “active sites” that can bind peptides and proteins and lower the sensitivity of the method. Even lo-bind equipment designed to reduce protein binding does bind some protein [22].

Reduction

The reduction of proteins is an important part of the sample preparation in bottom-up proteomics to increase the cleavage efficiency of the protease by making the cleavage sites more accessible, and prevent the proteins to aggregate [23]. The reduction is performed to break the disulfide bridge in cystines (pairs of cysteine residues), which are originally formed by oxidation of the thiol (–SH) group in the cysteine side-chain in the presence of another
thiol group. The reaction is catalysed and regulated by a series of enzymes in eukaryotes, but the thiol groups are highly reactive and are able to react spontaneously [23, 24]. The reduction process, which are typically performed with either β-mercaptoethanol (not very common anymore), dithiothreitol (DTT) or tributylphosphine (TCEP), reduce the disulfide bridge (-S-S-) to form the original free thiol groups (Figure 2) [8, 25]. Protocols are available for reduction in both gel and solution [22].

![Image of mechanism of reduction of disulfide bonds in proteins with DTT](image)

**Figure 2** Mechanism of the reduction of disulfide bonds in proteins with DTT. Every circle represents an amino acid, while R1 an R2 are continuations of the polypeptide/protein chain. In the first step, one of the thiol groups on the DTT forms a disulfide bond with one of the sulfurs in cystine and provides a hydrogen ion to form a thiol with the second sulfur atom in cystine. In the next step, a cyclic disulfide is formed by breakage of the recently formed disulfide bond between the cysteine cross-link and DTT by introduction of the second thiol group of DTT. The hydrogen ion forms a thiol group with the last cysteine sulfur atom.

**Alkylation**

Due to the reactivity of the cysteine thiol groups, the proteins are usually alkylated after reduction to prevent a re-oxidation [23, 26]. Commonly, the reaction is performed with iodoacetamide (IAA) as a separate step after reduction [8, 25], and follows a one-step bimolecular nucleophilic substitution (SN2) reaction mechanism to form a thioether that is much less reactive than the thiol group [26]. However, reagent mixtures with possibility of reduction and alkylation in a single step have been developed [23]. The alkylation process with IAA is illustrated in **Figure 3**, and shows the reaction mechanism; the nucleophilic sulfur in the thiol attracts the carbon atom (to form a bond) bonded to the iodide, which leaves
with the electron pair, while the hydrogen ion from the thiol group is lost. Still, the alkylation with IAA suffer from selectivity issues and the process can take place at other nucleophilic sites like primary (on the amino terminal (N-terminal) of lysine (Lys)) and secondary (histidine) amines, hydroxyls (tyrosine) and thioethers (methionine). Alkylation for 4-6 hours at basic (pH > 8) conditions retains a high selectivity, but the alkylation will often be incomplete. By increasing the alkylation time, a higher rate of unspecific alkylation reactions has been observed [26]. As a result, protocols have to compromise.

![Figure 3 Alkylation of a free cysteine thiol group in a peptide with IAA. R1 and R2 represent a continuation of the polypeptide chain. The nucleophilic sulfur in the thiol attracts the carbon atom bonded to the iodide, the hydrogen ion from the thiol group is lost, and the iodide leaves with the electron pair.]

**Proteolytic digestion**

Digestion is preferably performed with the protease trypsin, which cleaves proteins site-specific on the carboxyl-terminal (C-terminal) end of arginine (Arg) and Lys by hydrolysis [8, 17, 22, 27], and the reaction is seen in Figure 4. Good specificity of the protease is crucial to keep up with sample complexity in bottom-up proteomics, and is an important reason for the wide use of trypsin [8]. Nevertheless, there are a few general exceptions where the possibility of cleavage failure is larger. These include if the following amino acid to Lys or Arg is proline, if two or more Arg/Lys/protonated histidine follow each other (cleavage after the most C-terminal positively charged residue), or when deprotonated residues like aspartate/glutamate are close to Lys or Arg [8]. In addition, protein dependent random missed-cleavages will normally occur, making a “gold-standard” protocol hard to develop.
Figure 4 Proteolytic digestion (hydrolysis) with the protease trypsin after a Lys residue. R1 and R3 are both continuations of the polypeptide chain, while R2 is a side-chain group on a random amino acid.

Another benefit of trypsin is that Arg and Lys are frequently observed amino acids in mammalian proteins (6 % and 5 %, respectively) which give peptides with an average sequence length of nine amino acids [8, 22]. This normally creates information rich peptides with a convenient mass range (under 2 kDa), which give mass spectra that are easy to interpret. Nevertheless, it is important that only fully tryptic peptides are used with search engines for identification purposes to prevent false positive results [17]. Furthermore, trypsin is known to be a cheap, stable and proteolytic active protease providing high degree of cleavages [17, 22].

Other proteolytic agents are employed when trypsin cannot be used or to increase the cleavage coverage [28]. The endoproteases (cleaves the protein inside the polypeptide chain), Asp-N and Glu-C, are two examples of other proteases used, and they give peptides complementary to the peptides obtained with trypsin [17, 22]. However, the degree of cleavages is much smaller. Lys-C is another example which is often used before (or in combination with) trypsin under denaturing conditions due to its exceptional stability [17, 29]. In addition, chemical proteolytic agents, like cyanogen bromide, can be employed, but they are more commonly used as an addendum to digestion with trypsin to get more information about structural information such as post-translational modifications [8, 22].
2.2 Liquid chromatography – mass spectrometry

Today, LC-MS is often the preferable proteomic technique, mostly due to the high selectivity and sensitivity [7, 13, 30]. For the often highly complex samples in proteomics and bioanalysis in general, separation prior to MS detection is often of great importance. LC is a powerful separation technique, mostly used for non-volatile compounds. LC techniques use a liquid as mobile phase to carry analytes through a column with a stationary phase, which provide different degree of interaction with the analytes for their separation, before detection with a suitable detector. Generally, separation is important before detection of analytes in complex samples to improve the reliability of quantitative and qualitative determinations. In the case of MS detection, matrix effects due to high sample complexity can influence the sensitivity and hence give higher detection limit [31].

2.2.1 Mass spectrometry

MS is a technique for identifying the mass of a compound, or fragments of it. However, the compound/fragments must be in a gaseous phase and be charged (ion), because the MS separates ions accordingly to their mass to charge ratio ($m/z$). The MS generally consists of an ion source where the analyte is ionized (in most ion sources), a mass analyzer and a detector.

For LC-MS, the ion source is commonly soft, meaning that most ions reach the mass analyzer intact (not in fragments). The mass analyzer (also called mass filter) separates ions, before the $m/z$ ratios and their intensities are registered. In case of co-elution of compounds with the same mass (mostly in complex samples), fragment ions are often important for identification of the analyte. Hence, fragmentation of the precursor ions (intact ions without fragmentation) can be performed. How the fragmentation is performed depends on the mass analyzer, but the most common is to couple two mass analyzers (and a fragmentation cell), where one is used for selection of precursor ions and the other for separation of the fragment ions.

In the present thesis, a Q-Exactive MS consisting of both a quadrupole and an Orbitrap mass analyzer (the Orbitrap also works as detector) was mostly used (Figure 5). In the Q-Exactive, ions are transported from the ionization source to a beam guide separating ions from neutral molecules [9]. The ions are then separated in a quadrupole. This mass analyzer consists of four rod electrodes, where the opposite rods have the same potential (one pair with direct current and the other with alternating current) and ions are stabilized through the rods.
Because the potential needed for this stabilization is dependent on the mass and charge of the ions (a higher charge and mass require higher potential), only a small \( m/z \) value range will pass the rods and be detected at a given potential.

By doing a scan in time, where the potential is continuously increased, ions are separated in time. By selecting a range of potentials (or single potentials), only some of the ions will pass. In a single quadrupole, these ions will hit a detector which makes a signal with intensities corresponding to the number of ions hitting, and a mass spectrum appears.

**Figure 5** Sketch of the most important parts of the Q-Exactive MS. Ions are transferred through the beam guide to the quadrupole mass analyzer (also called mass filter), where ions or range of ions are selected. Ions are then transported to the C trap. In single MS mode, the ions are shot into the Orbitrap with the same kinetic energy, while in MS/MS mode, the ions are transferred to the fragmentation cell for fragmentation by a gas (\( \text{N}_2 \)) before they are transferred back to the C-trap and shot into the Orbitrap. In the Orbitrap, ions will oscillate back and forth between the two outer electrodes with different radius according to the \( m/z \) value. The oscillations induce image currents which makes a signal in time. Through an algorithm called Fourier transformation, the signal is translated to functions with frequencies corresponding to all the different \( m/z \) values in the Orbitrap. From these functions, the mass spectrum is easily made.

In the Q-Exactive depicted in **Figure 5**, selected ions from the quadrupole are transferred to the C-trap where all ions are relaxed. In MS mode, the ions are “shot” into the Orbitrap mass analyzer (with the same injection energy) for separation and detection. In MS/MS mode, the ions are transferred through the C-trap and to the higher energy collisional dissociation cell (HCD), where the compounds are fragmented by a collision gas (nitrogen gas) \[9\]. In data-dependent MS/MS (ddMSMS) mode, the Orbitrap only selects the base peaks (compounds with the highest intensities in the mass spectrum) for fragmentation.
Fragmentation of peptides generally produce several types of fragments (a, b, c and x, y, z), where the y and b fragments (Figure 6) are the most common (breakage of the peptide bonds) [22]. In principle, these ions give enough information in the mass spectrum to find the amino acid composition manually.

After fragmentation, the fragment ions are transferred back to the C-trap where all ions are relaxed, before they are “shot” into the Orbitrap mass analyzer. The Orbitrap consists of a center spindle electrode (some kV) and two outer electrodes (separated with an insulator) around. When ions are introduced; they will spin around the spindle electrode (oscillating back and forth) due to the electric potential, and induce an image current giving an alternating current as a function of time between the outer electrodes [32]. Because the spin radius is dependent on the m/z value, ions with different m/z values will induce current functions with different frequencies. Because all ions are oscillating at the same time, a signal corresponding to the sum of all the current frequencies is obtained, but the signal can be decomposed to the different frequencies by an algorithm called Fourier transformation [32]. The frequencies are easily converted into a mass spectrum. Due to the high mass resolving power (earlier called resolution) and mass accuracy of the Orbitrap mass analyzer, the Q-Exactive has shown to be excellent for peptide analysis (often complex samples with co-elution’s) [9].

**2.2.2 Electrospray ionization mass spectrometry**

For LC-MS, different ionization sources are available. Electrospray ionization (ESI) is one of them, and gained immediate popularity when demonstrated for use with MS. It is today the most frequently used ionization technique in proteomics due to its easy coupling with liquid phase separation techniques like LC or electrophoresis [5]. The analyte has to be mixed with a matrix (and dried and crystallized for matrix assisted laser desorption/ionization (MALDI))
before desorption with a laser/accelerated ions when using the ionization sources developed at about the same time as ESI; MALDI or fast atom bombardment (FAB) (see Textbox 1). On the other hand, ESI can create gas phase ions of easily ionized compounds (compounds with ionisable and polar groups) directly from the liquid form at atmospheric pressure, and can be coupled on-line with an LC-column [7, 22, 33, 34]. However, the solvent and buffer have to be volatile. This is important to have in mind when using ESI, but many of the common mobile phases and buffers in LC are (luckily) volatile.

The ESI process can be described as follows; first, charged droplets (of often already mobile phase charged analyte) are formed at the emitter/spray needle due to an applied electric potential up to several kV (typically up to 5 kV), supported by a nebulizing gas (usually N₂) and a drying gas in the opposite direction of the flow [34]. The potential, which is applied between the conductive emitter and the MS inlet, creates a large electric field because of the narrow emitter. For positive mode ionization, the MS inlet would be negatively charged relative to the emitter and attract the positive ions, while negatively charged ions are attracted to the oxidizing end at the emitter. Positive ions will also be attracted to the surface of the solvent while negatively charged ions move in the opposite direction [33]. Due to repulsive forces between ions of equal charge, the surface tension is overpowered and expands into a Taylor cone which further turns into a plume of droplets towards the MS inlet when the voltage is high enough [33]. For negative mode, the potential is reversed, but because the positive mode was used in the present study only positive mode will be discussed further.

The larger droplets formed after the Taylor cone are quickly reduced in size by evaporation of the volatile solvent and buffer due to the heated air around (higher thermal energy). Positively charged ions are still covering the droplet surface, moving towards the MS inlet. The evaporation increases the charge relative to the droplet radius, which at a curtain point overcomes the surface tension and leads to splitting of the droplets. This process is continuous, and is (among other) dependent on the current, conductivity of the solution, flow rate and the applied electric field. [33, 34]

For the last step, where small droplets with high charge result in gas phase ions, two theories are proposed. For larger molecules, such as peptides or proteins, the droplets are believed to split into smaller and smaller droplets as described above until only charged analyte in the gas phase are left. For smaller molecules, the analyte ions are believed to evaporate directly from the surface of the small droplets. [22]
Textbox 1

**Evolvement of ionization techniques for large biomolecules**

One of the most important reasons for the progress of mass spectrometry based proteomics was the evolvement of new ionization sources adapted to proteomic studies. In the beginning of peptide sequencing with mass spectrometry (in the 1960s), only one main ionization source; the electron ionization, was available [22]. With this ion source, the analyte had to be in gas phase before bombardment of electrons, requiring a volatile analyte. As peptides and proteins are large biomolecules made up of several amino acids bound together by a condensation reaction, the molecules are very little volatile. Nevertheless, peptide sequencing with gas chromatography coupled to mass spectrometry was performed with derivatives of very small peptides, often in addition to Edman degradation [22].

In the 80-90’s, the so-called soft ionization methods (little in-source fragmentation) made their entrance. In 1981, FAB was introduced, and in the 1990s, ESI and MALDI (**Figure T1**) followed [5, 22]. These ionisation sources caused a major breakthrough in MS-based proteomics, because they had the capability to generate gas phase ions of large, non-volatile molecules without decomposition of the molecule or extensive fragmentation [5, 7, 17, 22, 35].

**Figure T1** Principle of MALDI. A laser shoots at the dried and crystallized matrix. The laser desorbs and ionizes the matrix, carrying analyte particles (red). The analyte are then ionized by the matrix in the plume that is formed.
Nano-ESI

Nano-ESI (nanospray) is not considered a “smaller size ESI” according to Juraschek et al. and Scmidt et. al. [36, 37], even though the general principles are the same (but typically without nebulizing and drying gas, Figure 7). According to their studies, nanospray is advantageous over conventional ESI (ionspray) by means of lower sample consumption and detection limits, and higher tolerance for higher salt/buffer contents and polar solvents (such as water). The more highly charged droplets formed in the nanospray due to lower flow rates, increases the rate of droplet splitting. More frequent splitting decreases the concentration of salts-buffer per droplet compared to ionspray, thus a higher salt concentration in the solution can be used with nanospray. The fast splitting commonly gives ten times smaller droplets compared to ionspray already from the Taylor cone. The splitting process will further be increased because evaporation of solvent will happened faster for smaller droplets. In short, droplet formation is much more efficient. Because the charged analytes at the surface is more easily released from small droplets (higher charge to radius ratio), more ions are released into gas phase (giving lower detection limit). In addition, the emitter can be placed closer to the MS inlet due to the lower flow rates and increased droplet formation in the nanospray, decreasing the detection limit further.

Figure 7 Sketch of nanospray in positive mode. The liquid are sprayed through the needle tip by an electric potential. The liquid will form a Taylor cone and spread into a plume of droplets. The droplets will split into smaller and smaller droplets because of repulsive forces. This process continues until only the analyte ions in gas phase are left. These gas ions will be positive in positive mode and are attracted to the MS inlet.
2.2.3 Reversed phase LC-MS

Depending on analyte(s), several chromatographic principles can be employed for separation prior to MS. However, reversed phase (RP) LC is the most frequently used because of its excellent chromatographic efficiency and compatibility with electrospray ionization-MS (ESI-MS).

RPLC separates according to hydrophobicity. Consequently, a hydrophobic stationary phase is used with a mobile phase consisting of a pH adjusted mixture of water and an organic modifier (commonly methanol (MeOH) or ACN) [31, 34, 38]. Both isocratic (constant ratio of aqueous and organic solvent through the analysis) and gradient (increasing rate of organic solvent throughout the separation) can be employed. A gradient is commonly used for better separation when the analytes have very different retention on the stationary phase, even though the total cycle time will increase due to the need for re-equilibration (to ensure equal conditions in the column before each injection). A series of RPLC stationary phases are also available with either an organic, zirconia, titania or silica based support (e.g. particles) [34], and the main difference between the supports is that silica particles are known to be more mechanically stable (tolerate higher pressures), but do usually only tolerate pH values between 2 and 8.

Silica based particle types

Silica based particles (commonly 3-5 µm in diameter with a pore size of 100-300 Å) are the most frequently used [34, 39], and are available in two main types; totally porous particles and solid-core particles (also called core-shell, porous-shell, superficially porous particles etc.). While the porous particles are fully porous and usually with a smooth surface, the solid-core particles have a (often rough) shell covering a solid-core [39]. Usually, the shell is porous (Figure 8) because the pores increase the surface area, implying a higher loading capacity (maximum loading amount) than a nonporous particle. Hayes et al. [39] figures several types of solid-core particles available. Further, many methods for making

![Figure 8 Sketch of a solid-core particle type with porous shell (A) and a totally porous particle (B).](image)
these particles are also described in the literature [39, 40].

Because smaller particles increase both separation efficiency and backpressure (pressure drop over the column, see Appendix, section 6.1.1 for more details) [34], particles with high permeability (lower backpressure at the same flow rate) without losing efficiency is desirable. Traditionally, totally porous particles have been the standard particle type, but solid-core particles have been rising in popularity (especially for larger compounds like proteins) [41, 42]. Solid-core particles provide higher separation efficiency than porous particles (with the same particle diameter and under the same chromatographic conditions) because the diffusion of compounds in a thin porous layer contributes less to band broadening compared to the whole particle [34, 43]. Hence larger solid-core particles can be used with matching efficiencies to smaller totally porous particles, and higher flow rates (with equal pressures to totally porous particles) can be employed for faster analysis [39, 44-47]. Another reason for the higher efficiencies of solid-core particles is the lower size distribution for these particles, which gives a more densely packed wall region (implies better performance) [45, 46]. On the other hand, solid-core particles are more difficult to pack densely in very narrow columns due to the rough surface (particles “stick” to each other and less rearrangements will occur in a narrow column) [48].

Silica based stationary phases

Many different stationary phases are available for RPLC. Hybrid ligands with different functional groups to provide additional features and properties have increased in popularity in recent years [49], but n-alkyl ligands with endcapping (methylation of free silanol groups on the particle, to reduce secondary unwanted interactions) are still the most typical.

Among the n-alkyl ligands on silica, octadecyl (C$_{18}$) is commonly the ligand of choice (Figure 9). This stationary phase have been used for a wide range of applications regarding biomolecules, including proteins/peptides, drugs, metabolites, etc. [50-52]. C$_{30}$ is a less explored n-alkyl stationary phase than C$_{18}$ or C$_{8}$. However, C$_{30}$ has been known to provide more shape selectivity, meaning that the separation of compounds with different geometrical shapes (such as isomers or groups of similar compounds) usually is improved [53, 54]. Hence, C$_{30}$ has mostly been applied for determination of hydrophobic carotenoids (including isomers), which constitute a large group of similar structured compounds (organic pigments in plants) [53-55]. C$_{30}$ is mostly expected to give longer retention times than shorter carbon
chain stationary phases because of the increased hydrophobicity. However, C$_{30}$ has also proven to give different retention orders compared to C$_{18}$ in some cases, and some compounds could therefore have shorter retention times on C$_{30}$ [53].

Mobile phase additives

For ESI-MS purposes, a volatile additive (buffer or acid) is used to ionize the analytes prior to or in the ionization source to obtain high sensitivity. An ionized/charged analyte commonly implies a lower hydrophobicity and hence a lower retention on RP materials. In case of e. g. proteins and peptides, several additives can work as ion-pairing agents by binding to charged groups and consequently increase the hydrophobicity. Typically, trifluoroacetic acid (TFA) is used for this aim (Equation 1) to obtain sharper peaks with improved symmetry [56].

\[
\text{CF}_3\text{COOH} + M \leftrightarrow [M + H]^+ \cdot \text{CF}_3\text{COO}^- \quad \text{Eq. 1}
\]

However, TFA are known to cause ion suppression (lower signal intensity) because of the strong ion-pairing properties, and because the conductivity and surface tension of the water droplets formed in the ESI source increases [38, 57-60]. To cope with these issues, formic acid (FA) can be used to reduce ion suppression, but gives somewhat reduced chromatographic performance compared to TFA [31, 61]. Thus, several attempts have been performed to avoid this compromise [31, 58]. Addition of a surface tension reducing organic modifier (e.g 2-propanol or 2-(2-methoxyethoxy)ethanol) prior to the ESI source can enhance
the signal [59, 62]. The method called “TFA Fix”, developed by Apffel et. al [57], where both a signal-enhancing organic modifier and a competing weak acid working as a counter ion (e.g. propionic acid/2-propanol (75/25, v/v) in a 1/2 ratio (organic modifier/counter ion solution) to the LC flow) are added to the flow prior to the ESI source has showed the most promising results [31].

**MS detection**

Since proteins/peptides consist of both an N-terminal (if this end is not alkylated) and usually one or more amino acids with a substituent group (R-group) containing basic nitrogen sites, positive mode electrospray ionization will normally create molecular ions with a charge of 2 or higher (designated (M+nH)\(^{+}\), where M is the mass number of the analyte, H is the mass number of a proton, and n is a integer with a value of 2 or higher) [17]. Hence, the compounds mass number will commonly not be equal to the \(m/z\) ratio in the MS. This increases the complexity of the mass spectrum of peptides and proteins.

With a MS with high mass resolving power, such as an Orbitrap MS, the charge state can be derived from the cluster of isotope peaks [17]. However, in bottom-up proteomics, database searches based on different algorithms are commonly used. The algorithms use the \(m/z\) values obtained in the MS spectrum to find or connect to known/investigated peptides in databases. Because isomers and often different peptides have equal \(m/z\) values for a precursor ion, MS/MS can be used. The \(m/z\) values of the fragment ions are then used in the algorithm to confirm the identity of the peptide.

SEQUEST was the first algorithm for automatic database searches using MS/MS, and is still one the most common search algorithms used [63]. In the software, certain thresholds for the algorithm are made (e.g. mass tolerance, protease used during digestion of the proteins, ion mode (positive or negative), modifications, and the specific target protein or species which should be included in the search). The algorithm will then do an *in silico* digestion and fragmentation of the proteins fitting the criteria (with the selected protease), and compare the masses obtained in the MS/MS mass spectrum to the defined mass tolerance in the *in silico* digestion and fragmentation [64]. All matching masses will get a score mainly based on the number of matching fragment masses and their intensities, where a higher score imply a more reliable identification.
2.3 Chromatographic performance

2.3.1 Column band broadening

For isocratic elution, column efficiency is measured in the SI unit plate number (N), and is a measure of band broadening in the column. As seen in Equation 2 [32, 34], where N is the plate number, $t_R$ is the retention time, $\sigma^2$ is the band variance (time dependent and hard to measure experimentally), and $w_{0.5}$ is the peak width at 50% of the peak height, the band broadening increases with longer retention times.

$$N = \left(\frac{t_R}{\sigma_t}\right)^2 \approx 5.55 \left(\frac{t_R}{w_{0.5}}\right)^2$$

Commonly, the efficiency is given in plate height (H), which is inverse proportional to the plate number, and shows that the column efficiency also is dependent on column length (L) (Equation 3).

$$H = \frac{L}{N}$$

Band broadening is caused by several processes inside the column. Natural diffusion because of concentration differences inside the column will always occur (longitudinal diffusion), but depends on the flow rate (less diffusion if a high flow rate is used). When a compound enters the column in a small band, this concentrated band will diffuse into the zones around with lower concentration of the compound. In a packed column, particles will, because they are not packed perfectly inside the column, also lead to channels of different width and length between particles. Hence, some of the compounds travel faster than others (higher velocities in wider channels and lower velocities closer to the particles) and causes band broadening (eddy dispersion). This process is often considered the largest contributor to reduction in column efficiency, and is reduced with decreasing particle diameter. Band broadening is also due to transportation of compounds in the particle pores and convection between the stationary phase and mobile phase (resistance to mass transfer). In this process, band broadening increases (in a packed column) with increasing particle diameter, flow rate and reduced mobile phase diffusion coefficient.
Based on the above, packed column efficiency is solely based on the diffusion coefficient of the solute in the mobile phase, particle diameter and flow rate. The different contributors to band broadening in the column combined is commonly described by van Deemters equation, given in Equation 4 [34].

$$H = A + \frac{B}{u} + Cu = 2\lambda d_p + \frac{2\gamma D_m}{u} + \frac{f(k)d_p^2u}{D_m}$$  
Eq. 4

In Equation 4, $A$ is the eddy dispersion, $B$ is the longitudinal diffusion, $C$ is the resistance to mass transfer, $u$ is the flow rate, $\lambda$ is constant related to particle shape, $d_p$ is particle diameter, $D_m$ is the diffusion coefficient of a solute in the mobile phase, $\gamma$ is a constant and $f(k)$ is a function dependent on the retention factor.

Equation 2-4 are valid for isocratic elution, however they cannot be used for gradient elution because they depend on constant mobile phase composition. The retention factor, which is used to compare retention times and retention time stability, is also only valid for isocratic elution. For gradient elution, the efficiency may better be described by the PC (commonly $n_c$ but PC in the present thesis to reduce confusion). PC is described as the maximum number of peaks possible to separate in a given retention window (high PC implies a better separation, see Figure 10), and is given by Equation 5 [34, 65, 66].

$$PC = \frac{t_z - t_2}{w_{av}} + 1$$  
Eq. 5

In Equation 5, $w_{av}$ is the average peak width for all separated compounds in the gradient at 10 % (complete separation) or 50 % of the peak height, and $t_z$ and $t_a$ is the retention times of the first and last eluting compound, respectively. The last term in Equation 5 is often skipped in

![Figure 10 Sketch of two chromatograms showing a higher PC (A) compared to a lower PC (B) in a 30 min gradient. Chromatogram A shows a PC of 23, while than shows a PC of only 5. The difference is due to the much narrower peaks in A.](image)
calculations (including in the present thesis) because the first term usually is much larger. Theoretically, all peaks are equally wide with an optimal gradient [67].

However, equal peak widths are often not the case in practice. Hence, when many compounds are separated with a gradient elution (e.g. peptides), peak widths for some of the compounds with different retention in the gradient are measured and represent the average peak width.

In addition, the asymmetry factor ($A_s = b/a$ in Figure 11) can be calculated to evaluate peak tailing and fronting. While tailing is common and give information about how well a column is packed, fronting is typically caused by overload of the column [34].

### 2.3.2 Extra column band broadening

The performance of a chromatographic system is not only determined by the band broadening from the column. The total band broadening also depends on instrumental/system contributions, and is calculated by summarizing the peak variances from all the contributors when using isocratic conditions (Equation 6) [68, 69].

$$
\sigma_{total}^2 = \sigma_{column}^2 + \sigma_{capillary/tubing}^2 + \sigma_{injector}^2 + \sigma_{detector}^2 + \sigma_{other}^2
$$  
Eq. 6

In Equation 6, $\sigma_{total}^2$ is the total variance/band broadening observed and $\sigma_{column}^2$ is the variance contribution from the column (section 2.3.1). $\sigma_{capillary/tubing}^2, \sigma_{injector}^2, \sigma_{detector}^2$ and $\sigma_{other}^2$ are the variances originated from tubing or capillaries, injection processes, detector (e.g. flow cell) and other (e.g. unions or emitter), respectively. Because ESI-MS does not use flow cells, the contribution from detector is low. On the other hand, an emitter is used, and will be an additional source of band broadening compared to e.g. UV detection.

Generally, the contribution from extra column band broadening increases relative to that of the column when the column band broadening decreases [70] (e.g. smaller particles or small retention factor of the analyte (column variance is dependent on retention factor) [69, 71]) and for narrow columns (e.g. nano liquid chromatography (nanoLC) columns) [71, 72]. However,
reduction of extra column band broadening can be done in several ways. A steeper gradient can be used [69], and the number of connections, ID and length of tubings/capillaries should be kept at a minimum because higher volumes increases band broadening [68, 69, 71]. Nevertheless, limitations due to backpressure and clogging will occur if tubing inner diameter is too narrow. For most conventional LC systems, tubings/capillaries are used between the injector and column, and column and detector. In the present study, capillary tubing was only used to connect the injector and the nanoLC columns, hence reducing the band broadening. All fittings should also be tight and free of dead volumes by using correct sized ferrules and squarely cut capillary tubing (Figure 12).

Band broadening from the injector and connecting tubings prior to the column can also be reduced by refocusing of the analyte onto the column. For conventional columns, refocusing is performed by using a gradient starting at a low organic content. Analytes spread in a broad band in the tubing/injection process are then gathered at the entrance of the column because of retention to the stationary phase. Focusing time (elution time with low organic content before gradient start) can also reduce band broadening further. However, focusing time increases analysis time, and is not recommended if only small band broadening contributions from the tubing and injection process are observed. In nanoLC, refocusing is often (including in the present study) performed by trapping of analytes on an on-line solid phase extraction (SPE) column.

---

Figure 12 Sketch of connections of capillaries with square cut (A) and slanted cut (B).
2.4 Miniaturized liquid chromatography

2.4.1 Decreasing column inner diameter (nanoLC)

In recent years, the ID of LC columns has decreased from the standard 4.6 mm to 2.1mm ID conventional columns, and further down to 10 µm to 100 µm ID nanoLC columns [34, 73]. Large columns are still frequently used for routine analyses due to well suited instrumentation for these columns, robustness and easy handling. Narrower columns would need more careful treatment and optimized connections (tubing, unions etc.) to avoid extra column band broadening [74, 75]. Nevertheless, smaller ID columns provide other significant benefits.

Most importantly, downscaling of column ID increases sensitivity for concentration sensitive detectors [68, 76-78] (Figure 13).

![Figure 13 Dilution in chromatographic systems with a conventional (upper column) and narrow column (lower column). Both columns have the same axial dilution (band broadening). However, the increased ID of the conventional column gives an increase in radial dilution, which will give a decrease in signal intensity (lower sensitivity for concentration sensitive detectors). Figure adapted with permission from [78].](image)

The increased sensitivity is due to the reduced radial dispersion (commonly called radial dilution, D) in narrow columns and is described by Equation 7 [34], where $c_0$ is analyte concentration in the sample, and $c_{\text{max}}$ is concentration at peak maximum.

$$D = \frac{c_0}{c_{\text{max}}} = \frac{e^{\pi r^2 k} \gamma \sqrt{\pi L H}}{V_{\text{inj}}}$$

Eq. 7
Because neither the particle porosity ($\varepsilon_t$), retention factor (k), length (L), injection volume ($V_{\text{inj}}$) nor plate height (H) are affected by the column radius ($r_c$), Equation 7 can be reduced to equation 8, stating that $D$ is proportional to the squared column radius.

$$D \propto r_c^2 \quad \text{Eq. 8}$$

Equation 8 states that a twofold reduction of column radius results in a four time decrease in dilution, and theoretically, the same increase in sensitivity. The sensitivity of a narrow ID column ($d_{\text{small}}$) compared to a lager ID column ($d_{\text{large}}$) can be calculated (approximately) by the use of the sensitivity factor ($f$) given in Equation 9 [68, 79].

$$f \approx \frac{d_{\text{large}}^2}{d_{\text{small}}^2} \quad \text{Eq. 9}$$

Analytes present in lower concentrations can be detected using a smaller ID column if the same sample volume is injected, or lower sample volumes can be used without the loss of sensitivity, which is advantageous when the sample amount is limited.

To maintain the same linear velocity, the flow rate is reduced for smaller ID columns without increasing the analysis time. The resulting solvent reduction can be beneficial from both an economical and environmental perspective because lower consumptions of (possibly expensive and toxic) organic solvents are used [73, 74]. The flow also increases the efficiency of ESI because the spray needle can be placed closer to the MS inlet (smaller droplets are formed and more ions reach the MS, see Figure 7 in section 2.2.2 about nanospay), aiding sensitivity [36, 37].

Furthermore, smaller ID is more compatible with temperature gradients due to the lower thermal mass of the stationary phase and column body [34]. The low thermal mass implies a faster response to temperature, and hence avoids an unwanted radial temperature gradient, which can be a problem for larger ID columns. However, the narrow ID of nanoLC columns give rise to the need of a frit instead of filters (which are typically used for conventional columns) to retain the particles inside the column without increasing the band broadening.
2.4.2 Frits

Frits in capillary columns are often made by tapping the capillary end into silica particles before sintering with heat/flame/electric arc (Figure 14) [80-83]. These frits are quick to make, but often limits the mechanical strength of the fused silica capillary because the polyimide coating is affected by the sintering.

Another common frit making procedure is based on synthesizing a porous silica skeleton frit inside a fused silica capillary by polymerization (sol-gel method, Figure 15) [84-86]. Variations of the preparation methods have been published, but all are mainly based on a cross-linking reaction between Kasil (a potassium silicate solution) and formamide, where the reaction is initiated by heat. This method does not alter the polyimide coating on the fused silica tubing, and the mechanical strength is maintained.

However, both frit types discussed above are increasing the length of the column without having retaining properties (no stationary phase) and an additional emitter is needed for connecting to ESI-MS, both of which could be a source of band broadening. To possibly minimize dead volume resulting from connections, some groups today are packing New Objective capillaries with a fritted ESI-tip integrated in the end for direct electrospray [84, 87-89]. One of the studies used larger particles (with C18 stationary phase) as frit to minimize possible band broadening further (retaining frit) [89]. In the present study, fritted New Objective Pico Frit capillaries and two other frit making procedures was examined for possible benefits.
2.4.3 Large volume injections

Due to the low ID of nanoLC columns, lower flow rates must be employed to maintain the same linear velocity. The injection volume must also be reduced accordingly to avoid band broadening ($\sigma^2_{\text{injector}}$) and save time (time-consuming to transport large sample volumes to the column with flow rates in the nL range) unless refocusing is used (see Appendix, section 6.1.2 for more). Lower sample volumes imply lower sample amounts injected on the column and hence higher detection limits. To take advantage of the sensitivity of nanoLC columns, large volumes (several µL) can be injected in a short amount of time by analyte focusing with an on-line SPE-LC system. Figure 16 shows the on-line SPE-LC set-up used in the present study (other set-ups are available), and is described by Taylor et al. [90]; the sample is loaded onto the on-line SPE with a higher flow rate and water content compared to that of the analysis. A higher flow rate can be used through the on-line SPE column because of its short length compared to the analytical column (less backpressure). The analytes are focused on the on-line SPE, while non-retained solvent and polar contaminants go to waste. When the valve is switched, a “stopper” makes the flow go through the analytical column (path of least resistance), and the mobile phase flow for separation (isocratic or gradient) is started.

Figure 16 On-line SPE-LC system for large volume injections with nanoLC columns. A liquid flow will always follow the path of least resistance. Because the analytical column exerts a high pressure, the flow (with high water content) goes to waste when the valve is in the position seen in the figure. Because the SPE column is shorter than the analytical column, a higher flow rate can be used to load the sample (less backpressure), and the analytes are focused on the SPE. When switching the valve, the stopper exerts a larger resistance than the analytical column, and the flow will carry the analytes to the analytical column. In this valve position, the flow is reduced and the organic content in the mobile phase is increased (isocratic or gradient).
2.5 Column packing technology

NanoLC columns are usually more expensive than conventional columns, and are generally less robust because of easy clogging. Particles are easily accumulated due to the low ID, and will give increased backpressure build up over time [91]. Hence, the number of injections with a nanoLC column is limited even when sample clean-up is used (e.g. with on-line or offline SPE). In-house packing of nanoLC columns is more inexpensive than purchasing commercially available columns, and can also increase flexibility regarding choice of stationary phase and particle sizes.

Several packing techniques have been investigated, including dry packing where the packing is performed with gases like $N_2$, $H_2$ or Ar [92], packing with supercritical fluid CO$_2$ [6, 88, 93, 94] and slurry packing (packing with liquid). Several of the publications demonstrate increased separation efficiency for the dry packing methods and packing with supercritical fluid compared to slurry packing techniques [92, 95]. However, in more recent years, few reports using these methods have been published. Hence, slurry packing continues to be the most commonly used technique [6, 93, 96].

Slurry packing is typically performed by suspending particles in a slurry solvent (organic solvent), and is subsequently transferred to a reservoir and transported into a column housing (steel/glass/fused silica capillaries) using a packing solvent (pushing liquid) and high pressures [48, 80, 97, 98]. In this set-up, the packing solvent has been observed to be of higher importance than the slurry solvent [99]. In the present study, a packing bomb set-up is used. With this method, pressure is applied (with a gas) onto the slurry containing reservoir connected to the column housing. The applied pressure will push the slurry into the column housing. Hence, no packing solvent is used, and slurry solvents are therefore mostly discussed further.

A key basis for providing an efficient column, and a major challenge in column packing, is to obtain a uniform and densely packed bed with few defects and voids. This is especially challenging with regards to small particle sizes, narrow column inner diameters or long columns [80, 81, 93, 100], which are typical in today’s proteomics. Hence, effects of e.g. temperature, sonication, packing pressure, slurry concentration (particles/mL solvent), and slurry and packing solvents have been studied in detail [80-83, 87, 97, 99-101]. However, general rules for optimal packing conditions are hard to establish for all particle types and
packing set-ups, and founding optimized conditions are usually considered the bottleneck in packing technology [100, 102].

**Solvents**

Carbon tetrachloride has previously been a popular slurry solvent because of its ability to provide columns with excellent separation efficiency [99]. However, because of the toxicity of this compound, other solvents are preferred. In this regard, several groups have investigated different slurry solvents (and packing solvents). The most common slurry solvents examined have been tetrahydrofuran (THF) [46, 99, 102], acetone [83, 97, 99, 100, 102-104], 67/33 % hexane/acetone [80, 82, 87, 102], (water)/ACN [81, 87, 99, 102, 105], (water)/ isopropanol [80, 82, 87, 106] and MeOH [99, 107].

In 2012/2013, [87, 88] investigated the influence of slurry- and packing liquids for reversed phase materials. They found that a water/isopropanol (10/90 %) mixture as a slurry solvent (and water/ACN (10/90 %) as a packing solvent) yielded slightly better column efficiency and stability, compared to hexane/acetone (67/33 %, slurry and packing solvent) and ACN/isopropanol (40/60 %, with ACN as packing solvent).

Other recent studies have expressed the importance of agglomeration (reversible clustering) of particles in the slurry to avoid voids or gaps which will decrease column efficiency and possibly influence quantitative reproducibility [83, 102]. Because the term aggregation typically is used instead of agglomeration (which is the correct term [108]) in this field of science, aggregation is used in further discussions in the present thesis.

A study by the research group of James W. Joergenson investigated different solvents for the ability to give aggregation of particles in a suspension [83]. In general, they found that very unpolar solvents, like hexane, gave more aggregation than more polar solvents which give interactions with free silanol groups (on the particle). However, a decrease in aggregation was found when an additional solvent was introduced to the slurry (binary solvents). For reversed phase materials, hexane would bind strongly to the material, and an additional more polar solvent (e.g. acetone) is often used. MeOH was in their study found to give the largest degree of aggregation, compared to hexane/acetone (67/33 %) and acetone for solid-core particles, and was also observed to give the highest column efficiency. On the other hand, aggregation is proposed to cause trouble packing more narrow columns (< 25 µm ID), according to a
study by Kennedy and Joergenson [81]. It was observed that the packing of very narrow ID columns often terminated after a while, even though the solvent flow continued.

Nevertheless, optimization of slurry solvent with high column efficiency in mind is not the only challenge in packing technology. Particles should also be suspended in the slurry during the complete packing process, which is especially important for the bomb packing system used in the present study because only parts of the slurry are used to fill the capillary. Hence, the optimal solvent need to give both excellent column efficiency and keep particles suspended during the packing process. According to Blue et. al, aggregation properties (giving excellent efficiencies) does not necessarily give long sedimentation time [83]. A stable suspension is, according to Capriotti et. al, also important to prevent uneven distribution of particles in the column or a slow packing process [87]. Theoretically, sedimentation of a particle in suspension is given by Stokes equation (Equation 10).

\[
v = \frac{2r_p^2(d_1-d_2)g}{9\eta}
\]

Eq. 10

In Equation 10, \(v\) is the sedimentation velocity of the particle, \(r_p\) is the radius of the particle, \(\eta\) is the viscosity of the solvent(s), \(g\) is the gravitational constant, and \(d_1\) and \(d_2\) are the densities of the particles and solvent, respectively. According to the equation, a lower viscosity of the solvent, larger particles and high particle density compared to the solvent density will provide faster sedimentation rates, while equal densities of particles and solvent yields long sedimentation rates \((v=0)\) [99, 109].

When packing shorter columns or when using solvents giving high packing rates, long sedimentation time is less important [110]. Empirical data by e.g. Shelly et al. [101], and a mathematical simulation by Vissers et al. [97] indicates that a slow packing velocity is beneficial because of time for rearrangements. On the other hand, high packing rates (obtained by low viscosity solvents and high pressures) have also been seen to increase column efficiency because the kinetic energy of the particles is higher (due to higher speed) [102, 111]. A high kinetic energy can give the possibility of rearrangements of already packed particles to form a denser bed.

Nevertheless, optimization of slurry solvents used in the literature cannot be used directly to establish a method with the packing set-up used in the present study, because the packing solvents used in the literature probably influence the packing process and column efficiency.
Hence, most of the slurry solvents discussed will be investigated in the present study. The key findings about the slurry properties and the effects are listed in Table 1.

**Table 1 Effect of slurry properties on packing rate and separation efficiency**

<table>
<thead>
<tr>
<th>Slurry properties</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggregating properties</td>
<td>Increases efficiencies.</td>
</tr>
<tr>
<td>Solvent polarity</td>
<td>Less polar solvents are often more aggregating.</td>
</tr>
<tr>
<td>Mixture of solvents (binary slurries)</td>
<td>Decreases aggregation</td>
</tr>
<tr>
<td>Solvent viscosity</td>
<td>Lower viscosities gives faster packing and increased column efficiency</td>
</tr>
</tbody>
</table>

**Slurry concentration**

The slurry concentration has not been observed to affect the sedimentation rate significantly [83, 87], but is important for the resulting column efficiency [48]. However, optimal concentration is probably dependent on column inner diameter, slurry solvent [81, 87], and particle type [48, 100].

Bruns et al. and Blue et al. (with 0.9-1.9 μm and 1.1-2.7 totally porous and solid-core particles, respectively) observed that higher slurry concentrations tended to give better column efficiencies for all flow rates with both a computational investigation of the bed morphology (arrangements of the particles in the column) and LC analysis [83, 100]. It was also found that only the size of aggregates increased with higher concentration, while the number of aggregates relative to single particles did not change [83]. However, the effect of high slurry concentrations on column efficiency was less for solid-core particles and column diameter/particle diameter (d<sub>c</sub>/d<sub>p</sub>) ratios below 25 [100]. According to the study, the reason was that the slurry concentration affects the degree of size segregation and number of voids. Usually, particles provided by the manufacturers have a size distribution of the particles (not completely uniform sizes). During packing, smaller particles often fill voids in the packed bed. Because voids and loosely packed regions mostly are located closer to the wall (wall effects: particles are restricted by the wall and cannot move as freely as in the middle of the column) and particles are allowed to rearrange more in low concentration slurries, smaller particles are mostly found in the wall region. This creates size segregation inside the column, resulting in decreased column efficiency [46, 48, 100].
On the other hand, rearrangements did not occur as often with high slurry concentrations, with both less size segregation and an increasing number of voids in the column (decreased efficiency) as a result. However, because the contribution from voids at high concentrations up to a critical point is less crucial regarding efficiency, higher concentrations are beneficial in most cases. Since solid-core particles usually have smaller size distribution, there is less potential for size segregation (denser wall region) [46] and slurry concentration gets less important.

Both Bruns et al. and Blue et al. used columns with IDs of 30-75µm and pressures up to 2000 bar. In contrast, Kennedy and Jorgenson (5 µm particles) were actually only able to pack very narrow columns (<25 µm) successfully when decreasing the slurry concentration [81]. This could be due to low packing pressures (<345 bar at all times), particle type, or apparatus used, but recent studies have confirmed the correlation between clogging of small columns and high slurry concentration even with packing pressures up to 2000 bar [104]. On the other hand, [81] observed that for larger column ID (> 50 µm ID), higher slurry concentration also gave faster packing.

Table 2 offers a simple overview of the optimal concentration for different scenarios and trends discussed in this section, but the slurry concentration should preferably be optimized for the particle type used [48].

Table 2 Key findings regarding optimal slurry concentration.

<table>
<thead>
<tr>
<th></th>
<th>High slurry conc.</th>
<th>Low slurry concentration</th>
<th>Less important with slurry concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column ID ≥ 25 µm</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Column ID &lt; 25 µm</td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>d_s/d_p &lt; 25</td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Solid-core particles</td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Fast packing</td>
<td>x</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Temperature**

It has been shown that particle sedimentation (associated with column clogging and poor efficiency) decreased with higher temperature [87, 88]. At 70 °C, columns were packed at a faster rate and gave significantly lower plate heights for all slurries tested, compared to the columns packed in room temperature.
Packing pressures

In some studies, packing with relatively low pressures (170 and 375 bar, respectively) resulted in columns with reasonable stability and efficiencies [81, 87, 88] (PC of 144 for a 20 cm column [87]). They demonstrated that it is not an absolute requirement to pack columns with thousands of bar as performed by other research groups [80, 82, 83, 104], even though high pressures can be somewhat beneficial [83, 98]. Higher packing pressure increases aggregation and is recommended to extend at least 50 % of the operational pressures to obtain a stable packed bed [98], which is important for column efficiency and repeatability. Smaller particles and longer columns also require higher pressures to overcome the backpressure formed by the packed bed. However, more advanced equipment is needed to obtain thousands of bar. Studies also express the need of slow depressurization (up to several hours/overnight [80]) after packing to prevent gaps and axial particle expansion (particles are loosened).

Particle shape

Throughout the years with two particle shapes available (regular and irregular), it has been discussed how the particle shape influence column efficiency. Some studies did not find any significant differences, e.g. [112], while another observed that the particle shape giving the best column efficiency was dependent on particle size. Regular particles was preferred for particles < 5 µm, and irregular particles for sizes > 10 µm [111].

To sum up, to get the best efficiency or PC possible, all parameters have to be optimized for the specific system and particle type by packing and testing. This illustrates that packing columns does not have many universal guidelines for all packing scenarios; to quote LC pioneer James W. Jorgenson, “…column packing is an art rather than a science.” [83]. However, it seems like packing with small particles with narrow size distribution, high pressures, and low viscosity solvents with high aggregating properties is a safe way to get well performing columns. A summary of particle properties and the effect on packing discussed in this introduction is listed in Table 3.
Table 3  The effect of different particle properties (on packing) discussed in this introduction.

<table>
<thead>
<tr>
<th>Particle properties</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diameter</strong></td>
<td>Lower particle diameters are harder to pack densely in nanoLC columns, but do generally give higher efficiency.</td>
</tr>
</tbody>
</table>
| **Shape**           | If particles $< 5 \, \mu m$ in diameter: regular shape = higher efficiency  
                      If particles $> 10 \, \mu m$ in diameter: irregular shape = higher efficiency |
| **Type (porous/solid-core)** | Porous: Generally lower efficiency due to size segregation  
Solid-core: generally higher efficiencies. However, some reports lower efficiency when packing in nanoLC format. |
| **Density**         | Particle densities equal to solvent density implies longer sedimentation times (easier packing with pressure bombs). |
2.6 Aim of study

Due to often small sample volumes in proteomic studies, highly sensitive methods are required. NanoLC column can, due to the low ID, increase sensitivity of LC methods. However, commercial available nanoLC columns are expensive (around $1800 [113]).

The aim of the study was to develop a simple (common solvents and few steps) low-cost and efficient (≤1 hour) packing procedure for nanoLC columns (both standard housing and “PicoFrit” housing from New Objective). The goal was to obtain similar performance to commercial nanoLC columns.
3 Experimental

3.1 Chemicals

Solvents, test substances and reagents

Human serum albumin (HSA) (99 %), and acetate salts from luteinizing hormone releasing hormone (LHRH) (≥98 %), [D-Ala2] methionine enkephalin (Met-Enk) (≥97 %), [D-Ala2] leucine enkephalin (Leu-Enk) (≥97 %) and bombesin (≥97 %) were all purchased from Sigma Aldrich (St. Louis, MO, USA). This was also the case for protein digestion reagents; iodoacetamide (IAM), dithiothreitol (DTT), ammonium bicarbonate (NH4HCO3), urea (98 %) and Sequencing Grade Modified Trypsin (V511A, Promega Corp), and additives; trifluoroacetic acid (TFA) (≥99 %) and formic acid (FA) (≥95 %). Water used for digestion, dilution and desalting of peptides was “LC grade” water from Thermo Scientific (Waltham, MA, USA).

Water used in mobile phases and packing slurries was type 1 water (resistivity of 18.2 MΩ•cm at 25 °C) from a Milli-Q Integral purification system with Q-POD (0.22 µm filter) dispenser from Millipore (Darmstadt, Germany). ACN, acetone, MeOH and ethanol (EtOH) were purchased from VWR (Radnor, PA, USA), and hexane from Merck (Kenilworth, NJ, USA). Nitrogen gas used for the in-house packing apparatus (≥99.9 %) and as nebulizing gas/fragmentation gas in the MS (≥99.999 %) was purchased from AGA (Oslo, Norway).

Solutions

A 50 mM NH4HCO3 solution was made by mixing 0.40 g NH4HCO3 with type 1 water to a final volume of 100 mL. The 8 M urea/50 mM NH4HCO3 solution was made by mixing 4.80 g urea with the 50 mM NH4HCO3 solution to a final volume of 10 mL.

Solutions of LHRH (0.2 mg/mL in water/ACN/TFA (97.9/2/0.1, v/v/v)), bombesin (1 mg/mL in water/ACN/TFA (97.9/2/0.1, v/v/v)), Met-Enk (0.5 mg/mL in ACN/water (70/30, v/v)), Leu-Enk (1 mg/mL in ACN/water (70/30, v/v)), 1 M DTT, 1 M IAM, and trypsin (10 mg/mL in a 8 M urea/50 mM NH4HCO3 solution) were prepared by M.Sc. Tore Vehus.
3.2 Materials and equipment

Column bodies
Most in-house packed columns had fused silica capillary housings from Polymicro Technology (Phoenix, AZ, USA) with an inner diameter (ID) of 50 µm and an outer diameter (OD) of 360 µm. Pico frit column housings of 50 µm ID and 360 µm OD with integrated PicoTip emitter (10 µm ID with 5 µm silica particles (frit)) from New Objective (Woburn, MA, USA) were also used.

Frit materials
Frits were made either from “Kieselgel 60” from Merck (Darmstadt, Germany) with particle sizes ranging from 0.063 mm to 0.200 mm according to the manufacturer, or a “frit kit” consisting of Kasil 1 and 1624 (potassium silicates/water (29/71 (w/w)) and formamide for producing polymerized frits (Next Advance, Averill Park, NY, USA). Kasil 1624 has a SiO₂/K₂O ratio of 1.65 (w/w) and Kasil1 has a SiO₂/K₂O ratio of 2.50 (w/w).

Packing materials
All particle types used were spherical silica particles, but with different stationary phases. The particle types are given in Table 4.

Table 4 Packing materials. Their main properties and manufacturer. Conventional columns (4.6 mm ID x 150 mm) with the listed particles were emptied, and the particles were air dried before packing in nanoLC column housings.

<table>
<thead>
<tr>
<th>Particle type</th>
<th>Name</th>
<th>Stationary phase</th>
<th>Size (µm)</th>
<th>Pore size (Å)</th>
<th>End-capping</th>
<th>Carbon load</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid-core</td>
<td>Accucore</td>
<td>C₁₈</td>
<td>2.6</td>
<td>80</td>
<td>Yes</td>
<td>9 %</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Solid-core</td>
<td>Accucore</td>
<td>C₃₀</td>
<td>2.6</td>
<td>80</td>
<td>Yes</td>
<td>5 %</td>
<td>Thermo Scientific</td>
</tr>
</tbody>
</table>

Commercial columns
All commercial analytical, guard and on-line SPE columns were purchased from Thermo Scientific. A 75 µm ID x 150 mm fused silica column containing 2.6 µm Accucore C₁₈ particles with pore sizes of 150 Å (only available pore size in this format) was used for the comparison with in-house packed columns. A 75 µm ID x 20 mm PepMap100 C₁₈ nanoViper
containing 3 μm totally porous silica particles (pore sizes of 100 Å) was used as on-line SPE column at all times when investigating nanoLC columns (both in-house packed columns and the commercial analytical column).

For the investigation of stationary phases, a C$_{18}$ and a C$_{30}$ conventional 2.1 mm ID x 150 mm stainless steel column packed with the same particles listed in Table 4 were used. A 2.1 mm ID x 10 mm guard column packed with the same material (and stationary phase) as the analytical column, was coupled to the respective analytical column using an appurtenant “universal uniguard holder” (Therformo Scientific) for 2.1/3.0 mm ID guard columns.

For off-line sample clean-up, an Isolute 100 mg (3 mL) C$_{18}$-solid phase extraction (SPE) column from Biotage (Uppsala, Sweden) was used.

**Connections and couplings**

Polyetheretherketone (PEEK) tubings were purchased from IDEX Health and Science (Oak Harbor, WA, USA). Wherever PEEK tubing was used, all connections were made with “Supelco PEEK fitting one-piece fingertight nuts” (Sigma Aldrich) and ZU1C stainless steel unions from Vici Valco (Houston, TX, USA). The same unions were used for connections between fused silica capillaries/columns, but with the use of fused silica FS1.4 ferrules (Vespel/graphite) and steel nuts (1.4 mm) from Vici Valco. All fused silica columns were also connected to an ES 542 stainless steel nanobore emitter (20 μm ID x 40 mm) from Thermo Scientific using an “Upchurch PEEK Microtight® Connector Butt” with “MicroFingertight I Fittings” and an 360 μm ID “Upchurch Microtight® Tubing Sleeve” (all from Sigma Aldrich). For all two-column set-ups, 1.6 mm (1/16”) stainless steel Tees from Vici Valco were used. All connecting fused silica capillaries had an ID of 20 μm and OD of 360 μm (Polymicro Technologies).

**Containers**

All caps (Snap Ring Cap (11 mm)) and vials used for packing (1.5 mL Chromatography Autosampler Vials) and chromatographic analyses (0.3 mL Microvials) were purchased from VWR. Other containers used were 1.5 mL “Protein LoBind” and “Safe-Lock” tubes from Eppendorf (Hamburg, Germany).
Other equipment

A SpeedVac from Thermo Scientific (previously Savant) was used to concentrate/dry samples, a Grant-Bio Thermoshaker (model: PHMT, PSC-20, 20x2.0 mL) from Grant Instruments (Shepreth, Cambridgeshire) was used for all automatic mixing, and a DeltaRange analytical balance (model AE166) from Mettler-Toledo (Columbus, OH, USA) was the only balance used.

3.3 Column packing set-up

Columns were packed using an in-house made pressure bomb (Figure 17) as described by Rogeberg et.al. in the supplementary manual of the article “Separation optimization of long porous-layer open-tubular columns for nanoLC–MS of limited proteomic samples” [114]. The pressure bomb consists of a large steel nut and a steel platform where nitrogen gas enters the vial container through a tunnel. A capillary is connected with steel nuts (1.4 mm) and FS1.4 ferrules. The nitrogen gas exerts a pressure on the slurry, making it rise into the capillary.

An ultrasonic bath (Digital Ultrasonic Cleaner, model ATM-0.7-LCD) from ATU Ultrasonidos (Manises, Spain) was used to prepare all slurries, (degassing mobile phases for LC-MS), and for packing with heat. For packing with magnetic stirring, a blue magnetic stirrer bar (3 mm x 3 mm) from VWR and a Topolino magnetic stirrer from IKA (Staufen, Germany) were used.

In the production of frits, a Crème Brûlée torch from Jernia (Kolbotn, Norway) and a gas chromatography oven from Shimadzu (Kyoto, Japan) were used. Manual examinations of nanoLC columns were always performed with a microscope (W10x/20) from Motic (Hong Kong, China).
3.4 LC-MS instrumentation

3.4.1 MS detection

A Waters 2695 Micromass ZQ (single quadrupole) MS with a standard electrospray ion source from Waters, and a “Q-Exactive” MS (quadrupole-Orbitrap) with a “Nanospray Flex” ion source from Thermo Scientific was used for investigation of conventional columns and nanoLC columns, respectively.

Conventional C18 and C30 columns

The MS was operated in positive mode and set to record a total ion current (TIC) with a mass filter of 250-2000 Da. The MS parameters were optimized for LHRH using direct injection, and are given in Table 5.

Table 5 MS-settings for conventional C18 and C30 columns

<table>
<thead>
<tr>
<th>Voltages (V)</th>
<th>Temperatures (°C)</th>
<th>Nebulizing gas flow (N2)</th>
<th>Ion energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillary</td>
<td>2430</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cone</td>
<td>60</td>
<td>300 L/hr</td>
<td>4.5 eV</td>
</tr>
<tr>
<td>Extractor</td>
<td>3</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>Radio frequency</td>
<td>5.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NanoLC columns

The MS was operated in positive mode with a mass filter of 350-1850 Da. The method was set to record a total ion chromatogram (TIC) with a mass resolving power of 70 000, an maximum injection time of 120 ms and an automatic gain control (AGC) of 1 000 000, in addition to a ddMSMS with a mass resolving power of 35 000, 60 ms maximum injection time and AGC of 100 000. The capillary voltage was set to 1.8 kV.
3.4.2 Computer software

Mass Lynx from Waters was used in the investigation of conventional C\textsubscript{30} and C\textsubscript{18} columns for: obtaining chromatograms, extraction of signals from \( m/z \) values, peak integrations, and all measurements. For the investigation of nanoLC columns, all computer softwares were from Thermo Scientific. “Xcalibur™ Software” was used to obtain chromatograms and mass spectra, while “Proteome Discover 1.1.0” was used for peptide identifications (via database searches with the SEQUEST algorithm) and all calculations (e.g. PC and retention time stability). The mass tolerance selected for the SEQUEST algorithm was 10 parts per million (ppm) for the precursor ion and 0.6 Da for the fragment ions. Only peptides with 6 to 144 amino acids were included, and a maximum of two missed cleavages were allowed. Acetylation of any N-terminal (+42 Da) and oxidation of methionine (+16 Da) was accepted as modifications in the algorithm. Trypsin was used as the protease. Peak smoothing was in the software performed automatically.

3.4.3 Pumps/autosamplers

A 2695 pump from Waters (Milford, MA, USA) and an Easy-nLC1000 pump from Thermo Scientific (both with autosampler) were used for investigation of conventional columns and nanoLC columns, respectively.

3.4.4 Connections

Conventional C\textsubscript{18} and C\textsubscript{30} columns

The 2695 pump, with an outlet PEEK tubing of approximately 0.18 mm (ID) x 30 cm, was connected to another PEEK tubing with a dimension of 0.1 mm (ID) x 50 cm. This tubing was further connected to the guard- and analytical column (guard column directly coupled to analytical column). The end of the analytical column was further coupled to the single quadrupole mass spectrometer by 0.1 mm (ID) x 10 cm PEEK tubing. See Figure 18 for illustration.
NanoLC columns

A 20 cm (± 5 cm) fused silica capillary was used as outlet tubing from the Easy-nLC1000 pump and was connected to the on-line SPE column for the “two-column set-up”. The on-line SPE column was further connected to a Tee union where the column and fused silica tubing (to the waste valve inlet) was connected (see Introduction, Figure 16 in section 2.4.3 for details about the set-up). The column end was connected to the emitter (20 µm ID x 40 mm) using the “Upchurch PEEK Microtight Butt Connector” with a tubing sleeve (360 µm ID).

3.4.5 LC-system settings

Conventional C18 and C30 columns

The pump was set to a flow of either 0.25 mL/min or 0.10 mL/min. Partial loop injection was executed with volumes of 1 µL - 100 µL for the investigation of column loading capacity. A 30 minutes gradient elution was employed with mobile phases consisting of water/FA (99.9/0.1, v/v) (solvent A) and ACN/FA (99.9/0.1, v/v) (solvent B). Unless indicated elsewhere, the linear gradient was 3 % to 36 % solvent B without any focusing time. 15 min re-equilibration time with 3 % solvent B were employed after a 5 min cleaning step with 95 % solvent B. The ramp time between gradient and cleaning, and cleaning and re-equilibration
was 2 min, giving a total cycle time of approximately 40 min. Loading capacity and peak area were calculated from the 2 point smoothed extracted signals from the \( m/z \) values (± 0.20 Da).

**NanoLC columns**

The flow rate was set to 300 nL/min for the commercial 75 µm ID column (indicated optimal flow for this column), and 130 nL/min for 50 µm ID columns. Partial loop injection was executed with 1 µL injections (20 µL/min uptake). Three replicates (n) or more were performed at all times. A 30 minute gradient elution was employed with mobile phases consisting of water/FA (99.9/0.1, v/v) (solvent A) and ACN/FA (99.9/0.1, v/v) (solvent B). All mobile phases were degassed with the ultrasonic bath. Trapping of analytes in the pre-column was for all columns performed with 100 % solvent A for 10 min with a 500 nL/min flow (5 µL). The linear gradient was set to 3 % to 15 % solvent B, followed by a cleaning step up to 95 % solvent B for 15 min (15 % to 95 % B in 10 minutes). A 10 min pre-column equilibration (2 µL for all columns) and 25 min re-equilibration time (5 µL and 12 µL for the 50 µm ID and 75 µm ID column, respectively) with 100 % solvent A were used.

3.5 **Preparation of in-house made frits**

**Method 1, sintered frit**

Frits were made by tapping the end of a ~40 cm long fused silica capillary into “Kieselgel 60” until about 1 mm of the capillary end was packed (the frit). The frit length was inspected with a microscope and sintered with the Crème Brûlée torch for about 1 minute to attach the Kieselgel particles to the column wall. Finally, the polyimide residues were wiped off with a tissue with MeOH.

**Method 2, polymerized frit**

Kasil 1, Kasil 1624 and formamide were mixed to a final Kasil 1624/Kasil 1/formamide ratio of 3/1/1 (v/v/v) in a “Safe-Lock” tube. The fused silica capillary (~40 cm) tip was dipped into the solution for 3 seconds and put in the gas chromatography oven over night at 100 °C (about 15 hours) for polymerization. After polymerization, the capillary was flushed with ACN, and the frit was cut to a length of about 1-2 mm.
3.6 Column packing procedure

Packing with heated ultrasonic bath

Slurries (1 mL in total) were made by mixing solvent(s) with particles into a vial to a final particle concentration of 30 mg/mL, and the vial was capped. The suspension was sonicated with the ultrasonic bath at 65 °C (± 5 °C) for 10 min before the vial was placed inside the pressure bomb (uncapped), except for the hexane/acetone slurry which was sonicated at room temperature (see Appendix, section 6.2.3 for more details). A ~40 cm fritted capillary (with the frit/tip pointing upwards) was attached to the pressure bomb (airtight) with a nut and ferrule, with the other end of the capillary in the suspension (as shown in Figure 17 in section 3.3). The nitrogen gas flow was turned on (approximately 150-200 bar) and the bomb was placed into the ultrasonic bath at 65 °C ± 5 °C (maximum temperature tolerance of the particles is 70 °C) for 1 hour. The packed bed length was measured with a ruler before the pressure was released. For more details, see the standard operation procedure in Appendix, section 6.7.

For obtaining a denser packing, columns were connected to an EASY-nLC1000 pump and packed under isocratic conditions with ACN/water/FA (80/20/0.1, v/v/v) for 30 min at a flow rate of 200 nL/min. Columns were then cut to 15 cm and reconnected. The isocratic conditions were changed to water/ACN/FA (95/5/0.1, v/v/v) at 200 nL/min, and was employed until a stable pressure (400 +/- 75 bar) was obtained and until a stable electrospray in the MS had formed.

Packing with magnets

Column packing with magnets was performed in the same way as with heated ultrasonic bath, but the ultrasonic bath was not heated and was solely used for sonication of the slurry (25-29 °C) prior to the packing. To keep the particles suspended, a stirring bar was added to the slurry and the pressure bomb was placed on the magnetic stirrer during packing.
3.7 Sample preparation

3.7.1 Enzymatic digestion of proteins

Reduction, alkylation and digestion

The proteins were reduced and alkylated prior to digestion; 1 mg protein was dissolved in an 8 M urea/50 mM NH₄HCO₃ solution into a “Protein LoBind” tube to a final protein concentration of 1 mg/mL, and 1 M DTT was added to a final concentration of 5 mM (5 µL). After mixing and incubation for 37 °C in the Grant-Bio Thermoshaker, 1 M IAM was added to a final concentration of 15 mM (15 µL). The solution was mixed and incubated in the dark at room temperature for 30 min. The final solution was distributed into 10 “Protein LoBind” tubes.

The enzymatic digestion was performed by adding 50 mM NH₄HCO₃ until urea concentration was below 1 M (700 µL in each tube), and trypsin (10 mg/mL) was added to a protease/protein ratio of 1/20 (w/w) (0.5 µL into each tube). The sample was incubation with trypsin for 18 hours at 37 °C in the Grant-Bio Thermoshaker, before protease activity finally was inactivated by adding TFA to a final concentration of approximately 0.1 % (v/v) (pH<4) (1 µL in each tube).

Peptide desalting

Desalting was performed with the Isolute C₁₈-SPE column. The column was first conditioned with 1 mL ACN/TFA (99.9/0.1, v/v) and furthermore 3x1 mL water/ACN/TFA (97.9/2/0.1, v/v/v). The sample was applied and the collected eluent was reintroduced to the column before washing with 1 mL of water. The elution was performed by adding 1 mL ACN/TFA (99.9/0.1, v/v) into a “ProteinLoBind” tube. Finally, the eluent was dried using the SpeedVac.
### 3.7.2 Peptide test solutions

HSA (see Appendix, Figure 37 in section 6.3 for complete amino acid composition) was digested and desalted as described in the section 3.7.1 (‘‘Enzymatic digestion of proteins’’). For investigation of conventional columns, solutions of LHRH, bombesin, Met-Enk, and Leu-Enk were dried with the SpeedVac and redissolved in water/ACN/TFA (97.9/2/0.1, v/v/v) to a final concentration of 0.1 mg/mL. A mixture of the standard solutions was also dried and redissolved in water/ACN/TFA (97.9/2/0.1, v/v/v) to a final concentration of 0.1 mg/mL for all standards (mixture 1). Digested, desalted and dried HSA was redissolved in water/ACN/TFA (97.9/2/0.1, v/v/v) to a final concentration of 1 mg/mL (starting protein/solvent). Eight peptides spread in the gradient window were selected for PC calculations (Table 6).

**Table 6 Selected masses for PC calculations for conventional C{subscript 18} and C{subscript 30} columns.**

<table>
<thead>
<tr>
<th>Order</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/z</td>
<td>695</td>
<td>372</td>
<td>821</td>
<td>416</td>
<td>477</td>
<td>521</td>
<td>829</td>
<td>642</td>
</tr>
</tbody>
</table>

For investigation of nanoLC columns, digested, desalted and dried HSA was redissolved in water/ACN/TFA (97.9/2/0.1, v/v/v) to a final concentration of 1 ng/µL. Eight peptides (Table 7) were selected for all measurements (unless indicated elsewhere).

**Table 7 The eight selected peptides (from trypsinated HSA) with retention order and protonated mass (MH{superscript +}) for nanoLC column evaluation.** All sequences are listed with the one-letter code for the amino acids (see Appendix, Table 10 in section 6.3 for translation to amino acids).

<table>
<thead>
<tr>
<th>Order</th>
<th>Peptide sequence</th>
<th>MH{superscript +} (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>cASLQK (t{subscript a})</td>
<td>709.36</td>
</tr>
<tr>
<td>2</td>
<td>ADDKETcFAEEGKK</td>
<td>1627.73</td>
</tr>
<tr>
<td>3</td>
<td>ADDKETcFAEEGK</td>
<td>1499.63</td>
</tr>
<tr>
<td>4</td>
<td>EccEKPLLEK</td>
<td>1305.62</td>
</tr>
<tr>
<td>5</td>
<td>QTALVELVK</td>
<td>1000.60</td>
</tr>
<tr>
<td>6</td>
<td>QNcELFEQLGEYK</td>
<td>829.38</td>
</tr>
<tr>
<td>7</td>
<td>VFDEFKPLVEEPQNLK</td>
<td>2045.09</td>
</tr>
<tr>
<td>8</td>
<td>ALVLIARAFQYLQQcPFEDHK (t{subscript a})</td>
<td>2490.29</td>
</tr>
</tbody>
</table>
3.8 Statistical evaluations

In the present study, a two-sided “Grubbs’ test” (test of outliers, chapter 3.7 in [115]) was generally used to detect outliers with four or more replicates. Data found to be significant outliers according to $P > 0.05$ were rejected in all calculations and in most figures (unless indicated elsewhere), when the data was found to be normally distributed according to a “Shapiro-Wilk test” (when $W \geq W_{\text{critical}}$ at 5 % significance level) [116]. Unpaired two-sided “Student’s t-tests” (comparison of two means including F-test for comparison of standard deviations, chapter 3.3 and 3.6 in [115]) with significance level 5 % ($P > 0.05$) were used get an indication [117] of significant differences between data sets.
4 Results and discussion

In the present study, optimization of in-house packing of nanoLC columns has been the main focus. In the optimization, different packing approaches (heat and magnetic stirring), slurry solvents, frit types, and column housings have been compared to find the easiest/simplest procedure, which creates the best performing columns with regard to PC and retention time stability for peptide separations. In addition, investigations of different stationary phases (C\textsubscript{18} and C\textsubscript{30}) were compared for peptide analysis.

4.1 Investigation of conventional C\textsubscript{18} and C\textsubscript{30}

Prior to optimization of packing procedure, a stationary phase suitable for peptide separation had to be selected. Hence, conventional columns with C\textsubscript{18} and C\textsubscript{30} were compared for peptide analysis regarding fundamental chromatographic properties. The flow scheme below shows an overview of the chromatographic properties investigated for both stationary phases.

C\textsubscript{30} vs C\textsubscript{18} for peptide determinations

- **Retention times**
  - were compared to investigate whether the more hydrophobic chains of C\textsubscript{30} could increase the retention times for better separations

- **Peak capacities**
  - were compared to investigate whether C\textsubscript{30} improves separation efficiency

- **Loading capacities**
  - were compared regarding peak width, asymmetry and peak area, to investigate whether more sample could be loaded on to a C\textsubscript{30} stationary phase
4.1.1 Retention time comparison for peptides

C\textsubscript{30} and C\textsubscript{18} are well known stationary phases in reversed phase chromatography. However, few studies report the use of C\textsubscript{30} for peptide separations. Comparisons of fundamental chromatographic properties (PC, retention time and retention order) were performed in a preliminary study to select stationary phase for peptide analyses with nanoLC columns.

Both columns contained Accucore (solid-core) particles, which have showed promising performance \cite{118} and satisfactory packing properties (see Appendix, section 6.2.2). A conventional flow rate of 0.25 mL/min was selected \cite{119}. Higher flow rates were not used because of pressure restrictions. To scout for advantages of lower flow rates \cite{34}, 0.10 mL/min was also examined. All investigations were performed with 5 μL injections of four peptides; bombesin, Met-Enk, LHRH and Leu-Enk, and a 30 min 3-36% solvent B gradient (recommendations from application note for Accucore columns \cite{120} and M.Sc Tore Vehus). Up to 10 min focusing time, which could increase the refocusing effect of analytes and hence increase PC, was investigated. However, because no difference in PC was observed for the chromatographic system used, focusing time was not used further. 15 min of re-equilibration was considered sufficient for repeatable chromatography.

In the present study, only modest changes in retention time and no differences in retention order was observed on C\textsubscript{30} compared to C\textsubscript{18}. At 0.25 mL/min, retention times on C\textsubscript{30} increased by an average of 5.3 % ± 0.8 % for all peptides (with an average of 1 % variance between replicates) compared to C\textsubscript{18} (Appendix, Table 14 in section 6.5.1). For 0.10 mL/min, retention times on C\textsubscript{30} increased by an average of 10 % ± 2 % (Appendix, Table 15 in section 6.5.1). No variance between replicates is reported for the 0.10 mL/min flow because only one replicate per standard was used for this flow in this preliminary study.
4.1.2 Peak capacity

The PC (i.e. maximum number of peaks that can be separated in a given time) for the C\textsubscript{30} and C\textsubscript{18} column was also compared under the same conditions as for the retention time examination, but with 5 \textmu L injections of peptides from digested HSA. HSA was used in this regard to increase both retention window and number of peaks for more accurate PC measurements.

No significant difference in PC at 10\% of the peak height (PC\textsubscript{10}) was observed between C\textsubscript{30} and C\textsubscript{18} for any of the flow rates (Appendix, Table 16 in section 6.5.1), but the PC\textsubscript{10} using a flow rate of 0.10 mL/min increased by 9 and 11 (64 \% and 73 \%) compared to 0.25 mL/min for C\textsubscript{18} and C\textsubscript{30}, respectively. Nevertheless, 0.25 mL/min were selected for loading capacity investigations because of the much shorter retention times and somewhat more symmetric peaks for peptides from HSA digest as seen in the chromatogram in Appendix (Figure 44 in section 6.6).

4.1.3 Loading capacity

The loading capacity for C\textsubscript{18} and C\textsubscript{30} materials was compared to investigate whether larger sample amounts could be loaded on the C\textsubscript{30} material than the C\textsubscript{18} material without decreasing column efficiency by column overloading as indicated by [121] with LC-NMR. The comparison was performed by varying the injection volume of mixture 1 (see Experimental, section 3.7.2) from 1 \textmu L to 100 \textmu L (0.1 \textmu g to 100 \textmu g), and calculating peak width, asymmetry, and peak area.

Increasing the injection volume generally led to an increase in peak width and asymmetry (although no change was observed for bombesin), which are signs of overload [122]. On the other hand, no significant differences between the C\textsubscript{30} and C\textsubscript{18} materials were observed.

Leu-Enk had the largest differences in both asymmetry (Figure 19A) and peak width (Figure 19B) between C\textsubscript{30} and C\textsubscript{18}, and the largest increase in peak width (161 \% and 136 \%) increase from the 1 \textmu L injection to the 100 \textmu L injection for C\textsubscript{18} and C\textsubscript{30}, respectively) (Appendix, Table 17, section 6.5.1) and asymmetry (145 \% and 150 \%) increase from the 50 \textmu L injection to the 100 \textmu L injection for C\textsubscript{18} and C\textsubscript{30} respectively) (Appendix, Table 18 in section 6.5.1). No carry-over was found after a 10 \textmu L sample injection.
Figure 19 Asymmetry (A) and peak width (B) at 10% of the peak height as a function of injection volume with 0.1 mg/mL Leu-Enk. The green line (A) shows the value 1, which indicates absolute symmetry. Values above 1, indicates tailing, and values below indicates fronting. The error bars shows the absolute standard deviation (STD) (n = 3 injections for all injection volumes on both columns). Leu-Enk was chromatographed with a gradient of 3% - 36% solvent B in 30 min, with a flow rate of 0.25 mL/min on the conventional C18 (blue) and C30 (red) column (both 2.1 mm ID x 150 mm with 2.6 µm Accucore particles).

Regarding peak area, the same trend was observed for all peptides on C30 and C18 when increasing the injection volume/amount. Peak area as a function of sample loading amount on both stationary phases followed a common second-degree polymeric trend, except for a significant inflection point for the 0.0050 mg or 0.0025 mg injection on C18. This point was considered an outlier due to system instabilities. Figure 20 shows Leu-Enk, which had the largest peak area, as an example.
Figure 20 Peak area as a function of injected sample amount (mg) of 0.1 mg/mL Leu-Enk separated on C\textsubscript{18} (blue line with stars) and C\textsubscript{30} (red line with squares). Error bars show the scattering, $w. n \geq 2$ injections for all injected amounts (injection volumes). Other conditions as described in Figure 19.

To summarize, differences in both peak widths, asymmetry and peak area between various peptide species were observed. However, C\textsubscript{18} and C\textsubscript{30} behaved mostly in the same manner regarding all the fundamental chromatographic properties examined (as proposed by Kalman et al. for other peptides in 1992 [123]). The retention order of peptides from HSA was the same, implying that co-eluting peaks in complex samples with HSA would interfere in the same manner for both phases.

A possible explanation for the similarity between the phases is the higher carbon load (number of bonded phases) of the C\textsubscript{18} particles (9\%) compared to C\textsubscript{30} (5\%), in practice outweighing the longer chains of the C\textsubscript{30} phase.

*Nevertheless, in this study, no large differences were observed, and the more common C\textsubscript{18} was chosen to be used in the optimization of nanoLC columns.*
4.2 Optimization of nanoLC column packing

For the optimization of nanoLC columns, different packing approaches, slurry solvents, frit types and column housings were investigated for the ability to separate peptides. The optimal packing procedure was selected based on the simplicity, packing rate (shorter packing time) and column performance. A flow scheme of the process and main results is presented in Figure 21.

**Figure 21 Flow scheme of the optimization of nanoLC column (50 µm ID) packing.** Column lengths were measured before depressurization (in the pressure bomb) with a ruler. In the LC set-up, the same commercial online SPE column was employed (75 µm ID x 20 mm), and a gradient of 3-15 % solvent B in 30 min (130 nl/min flow) was used with in-house packed columns cut to 150 mm. Hex/ac = hexane/acetone.

**Green checks:** Highest means

**Red checks:** Acceptable, but significantly lower mean than green checks

**Red X:** Not acceptable mean/poor total result
4.2.1 Comparison of column packing simplicity

A slurry packing method was used to pack all nanoLC columns, and was performed by filling a fused silica capillary with a slurry (mixture of particles and solvent) using a pressure bomb system. Due to the ease of the preparation of the polymerized frit, this frit type was used as the “standard” for all packing. In the packing set-up used, a key to success was keeping particles suspended in the slurry during packing (Figure 22). Two packing approaches (magnetic stirred slurry and heated slurry) for keeping particles in slurry were evaluated, focusing on their ability to pack at least 15 cm long columns (typical length of analytical columns) in a time frame of approximately 1 hour with high repeatability. Based on the requirements described above, the comparison was performed with a concentration of 30 mg Accucore C18 particles per mL solvent (isopropanol, an hexane/acetone mixture (67/33, v/v) and aqueous solutions of ACN, EtOH, and MeOH (organic solvent/water, 80/20, v/v) [124]). The slurry concentration used has been found to give good performance for 50 µm ID columns [100] and the efficiency has not been reported to increase by raising the concentration to 50 mg/mL for solid-core particles (used in the present study) [104]. The packing was always performed with at least 10 cm longer capillaries (~40 cm in total) than the expected packed bed length to avoid particles being lost during depressurization of the pressure bomb.

Heat-packing

Sonication is in most studies used for mixing and suspending particles in the slurry prior to packing. Sonication during packing has also been observed to give more stable packing over time and fewer voids [103, 105]. After 1 hour of packing with sonication and heat (65 °C ± 5 °C) it was observed that all particles had settled, indicating low effect from the sonication through the pressure bomb. Heat keeps particles suspended for a longer time (as seen in the initial sedimentation tests in the Appendix, sections 6.2.1 and 6.2.3).
Heat should also increase packing velocity due to reduced viscosity at higher temperatures [125], and could possibly increase the column efficiency [87, 88].

According to the scatterplot in Figure 23, which depicts packing lengths obtained with the heat-packing approach for the different solvents, only packing with 80 % ACN gave columns with satisfactory length (mean value) despite featuring one of the fastest sedimentation rates (see Appendix, section 6.2.3). The long column length is probably due to the low viscosity of 80 % ACN [126, 127], giving a faster packing velocity [111].

Nevertheless, the column length repeatability was poor, with a relative standard deviation in % (RSD%) of 25 % for 80 % ACN (n=10 columns). This can possibly be explained by changes in slurry concentration and/or composition due to solvent evaporation. The organic solvents will evaporate faster than water in the various steps before pressurization (e.g. during sonication and connection to the pressure bomb). Evaporation will be more significant with higher temperatures, and increased the viscosity (and hence decrease packing rate) due to increased water content [127].

The actual room temperature also affects the temperature in the heated ultrasonic bath, as the accessible (and aging) bath struggled to keep 65 °C at low room temperatures. Lower temperatures have in sedimentation tests (Appendix, sections 6.2.1 and 6.2.3) been shown to result in faster sedimentations, which would give a decrease in “input” slurry concentration. Lower slurry concentrations are known to give slower packing rates [104]. Lower temperatures would also increase the viscosity, and hence possibly decrease packing rates.

![Figure 23](image_url)

Figure 23 Length of packed material (cm) vs slurry solvent when packing with heated ultrasonic bath. All columns were packed (without any re-connections) for 1 hour. 10 minutes sonication (65 °C, except for hexane/acetone) of the slurry was performed prior to packing. All points show the packing length of individual columns, and the lines show the average packing length. n≥3 columns for all slurry solvents.
Variations in sedimentation level before pressurization in the pressure bomb would also decrease the repeatability because the time from sonication to pressurization varied.

Other general disadvantages with the heat-packing approach were the high temperatures needed (safety precautions), and the need of heating water prior to packing.

**Packing with magnetic stirring**

With magnetic stirring, particles were suspended during packing, easing the packing somewhat by eliminating the need of heat. The particles were also not completely settled after the 1 hour cut-off, and the effect of this can be seen in the scatter plot in **Figure 24**, where the packing length is clearly longer than for the heat-packing approach seen in **Figure 23** (from 44 % to 156 % longer columns depending on solvent).

![Figure 24](image)

**Length of packed material (cm)** plotted against the slurry solvent when packing with magnetic stirring. All columns were packed (without any re-connections) for 1 hour. 10 minutes sonication (25-29 °C) of the slurry was used prior to packing. All points show the packing length of individual columns, and the lines show the average packing length. \( n \geq 3 \) columns for all slurry solvents.

The column length repeatability was higher for the magnetic stirring method, but still there was a 9 % packing length variation for columns packed with 80 % ACN. The variations were most likely due to random errors, differences in room temperatures which would give small viscosity variations (columns were packed on different days), or sedimentation levels before pressurization as for the heat-packing approach.

Sedimentation was less of an issue with the stirring technique, but despite the use of magnetic stirring, some sedimentation did actually occur. Hence sedimented particles prior to pressurization were not easily re-suspended inside the pressure bomb, and lowered the concentration of particles in the suspension accessible to the column (lower packing rate as described earlier).
Despite longer packing lengths for the magnetic stirring method, only 80 % ACN and hexane/acetone (67/33, v/v) gave columns with the satisfying length (\(\geq 15\) cm) for all replicates. However, with the stirring method, all slurry solvents could be used by increasing the packing time slightly beyond 1 hour (only by 10-15 min) because suspended particles were left after 1 hour packing. It should be emphasized that even if particles are left in the slurry, the packing will eventually terminate after a while because a constant pressure was used (see Appendix, section 6.4.1 for more details).

All slurry solvents managed to provide 15 cm packing length with magnetic stirring and were further investigated for the ability to provide columns with high PC. The columns packed with 80 % ACN with magnetic stirring and the heat-packing approach (the only slurry solvent providing >15 cm with this approach) were compared to investigate any advantages heat may give regarding column performance, as proposed by literature [88].

However, the stability of the packed bed was investigated before the nanoLC columns could be used for further investigations, because focusing of the particles in the column after packing with the pressure bombs was observed to be necessary.

### 4.2.2 Stability of the packed bed

The stability of a nanoLC column packed with Accucore C\(_{18}\) particles was investigated by observing the particles inside the column under the influence of operational pressures (at a flow rate of 130 nL/min) directly after packing with the pressure bomb.

Post-bomb packing with an LC pump was observed to be necessary to obtain a dense particle bed without movement of particles. However, some particles were lost at the column inlet (with no frit) when the LC pressure was released. Table 8 shows how the higher pressures of the LC pump gathered remaining clusters (loosely packed small groups of particles) inside the column after packing with the pressure bombs. Hence, a washing step with 80 % ACN (at > 350 bar) was included before cutting of the column to 15 cm. The washing step also removed remains of possible contaminants and slurry solvents from packing. A mixture of 5 % solvent B and 95 % solvent A was used to obtain a stable spray in the nanoESI after cutting. Because no significant advantages were found by packing with an LC pump with high pressures for a longer time, no further packing was considered required (see Appendix, section 6.4.2 for more details).
The operational pressure was found to be sufficient for the focusing of particles. A flow rate of 200 nL/min was therefore employed only for a faster cleaning of the column. On the other hand, a loss of approximately 1 mm of the packed bed was observed after cutting and use (see **Appendix**, section 6.4.3 for more details).

**Table 8 The column outlet (frit end) and middle after packing with the pressure bombs and further packing with an LC pump.** All clusters inside the column were gathered after packing with the LC pump, and did not move significantly when the pressure was lowered back to atmospheric pressure. The in-house packed column (50 µm x 150 mm) depicted was packed with magnetic stirring and 80 % ACN as slurry solvent in a polymerized frit column housing.

<table>
<thead>
<tr>
<th></th>
<th>Front</th>
<th>Middle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column after packing with pressure bombs</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td>Column after post-bomb packing with an LC pump</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
</tbody>
</table>

**4.2.3 Column performance**

The nanoLC columns packed with the two packing approaches (heat and magnetic stirring) and the different slurry solvents was further investigated to find the packing conditions that gave the best performing columns in terms of PC and retention time repeatability (an important feature for peptide identification purposes). Two neat packing solvents, 100 % MeOH [83] and 100 % ACN, were also included in the investigations because of the possible increase in performance according to [83] (published after the time of the packing length experiments). In addition, sintered frits in fused silica capillaries and New Objective column housings were compared to the “standard” polymerized frit column bodies. The best performing in-house packed column was then compared to a commercial column (75 µm ID) packed with the same particles to investigate whether the in-house packed nanoLC columns matched the commercial one.

Peak capacities were calculated based on an optimized gradient of 3-15 % solvent B in 30 min. This gradient ensured peptides from tryptic HSA to elute in the later parts of the gradient window, in contrast to the previously used 3-36 % solvent B. In the nanoLC column pump system, fused silica capillaries with lower ID than the column was used to minimize band broadening from the tubing, and to reduce system delay. Because narrow 20 µm ID
connecting capillaries were used, the on-line SPE column (75 µm ID) was the main source of band broadening and system delay (only 20 mm of the 15 cm in total was packed with particles). Compared to the commercial column (75 µm ID), the narrower ID in-house packed columns (50 µm ID) were more affected by the system delay from the on-line SPE column due to lower flow rates. The flow rate used for the commercial nanoLC column (300 nL/min) was selected based on recommendation from the manufacturer, and the same linear flow according to the sensitivity factor was therefore used for the 50 µm ID columns (approximately 130 nL/min). The total system delay was therefore calculated to be at least 5 min with a flow rate of 130 nL/min.

Eight peptides from trypsinated HSA were selected for further measurements (Experimental, Table 7 in section 3.7.2) were selected based on retention time (to fill the gradient window). The first and last peptide observed with a clear peak and low noise (in the extracted ion chromatogram) for the first column was selected as $t_1$ and $t_s$.

Because of the ddMSMS mode, the last eluting peptide was in some replicates not identified. In order to calculate PC, the retention time of this peptide was required. Hence, approximate retention time was obtained from another replicate (1 ng/µL or 100 ng/µL) because the retention time variation between replicates did not give significant differences in PC. The peak width of (occasionally) not identified peptides was also excluded in the calculation of PC for the given injection replicates. This affected the average peak width, which can have given rise to some PC variations between replicates. However, this influence on PC was modest because the excluded peaks generally had similar bandwidths as the detected peaks. When only 5 or less peptides were found in one replicate, the replicate was rejected due to lack of data points for representative average peak width calculations.

A re-equilibration with 5 µL and 12 µL solvent A for the 50 µm ID and 75 µm ID columns, respectively (equivalent to 17-18 times the column volume) was considered sufficient for repeatable chromatography (Figure 25). The selected cleaning step between injections (95 % solvent B) was also sufficient to avoid carry-over when injecting 1 µL cell lysate samples [128]. All chromatograms were recorded with ion intensity measured in relative abundance in % (default setting), since no quantifications were made.
Figure 25 EIC of seven of the eight selected peptides from digested HSA (1 ng/µL) for three replicates of column 5 packed with 80 % ACN (intra-column retention time repeatability). Extracted signals from m/z values (with a 7 point peak smoothing) from left to right in the EIC: 353.68, 543.25, 750.32, 653.32, 500.81, 829.38, and 682.37. The m/z = 750.32 is hardly visible, and the peak around 33 min is an unknown compound with equal m/z to 500.81. The chromatograms are showing n = 3 injections (1 µL) for an in-house packed column (50 µm ID x 150 mm with 2.6 µm Accucore particles) packed with magnetic stirring in a polymerized frit column housing. The selected time frame was 5-35 min (gradient when delay is countered for). Separation was performed with a 30 min gradient of 3-15 % solvent B, and a flow of 130 nL/min.

Slurry solvents and packing approaches

For the magnetic stirring method, columns from all packing solvents were investigated because most columns were longer than 15 cm after 1 hour packing. Furthermore, because of uncomplete sedimentation after 1 hour, the remaining columns (bed < 15 cm after 1 hour) were packed with the pressure bomb until required length (approximately 1 hour and 10 min in total).
Comparison of slurry solvents

PC at both 10 % and 50 % of the peak height was calculated for columns prepared with the five main slurry solvents (Figure 26 A and B, respectively). In general, poor repeatability and small differences was observed regarding the PC for all the slurry solvents. As seen in Figure 26, the RSD% of PC generally ranged from 7 % to 18 % (both 10 % and 50 % of peak height) depending on slurry solvent. Isopropanol was extreme in this regard, with a RSD% of 25% (50 % of peak height) and 32 % (10 % of peak height). This large variation in PC is mostly due to two poor injection replicates for one of the three columns packed with isopropanol. For both of these two replicates, especially long tailing was observed for all peptides. Because the third replicate on the same column was “normal”, the reason was not connections or cutting of the column. However, the packed bed could have been unstable because the poor replicates were the first two replicates for this column. Otherwise, because the trend was similar for 50 % of the peak height and variations generally tended to be lower at this peak height, most calculations regarding repeatability and variations discussed further were done at 10 % of the peak height.

According to a t-test, the PC for columns packed with 80 % ACN was significantly higher than for the others (except for 80 % EtOH measured at 10 % of the peak height) with mean PC of 89 and 204 (at 10 % and 50 % of the peak height, respectively) for all replicates regardless of column (see Appendix, Table 20 in section 6.5.2 for example of calculations). However, the column performance variations between slurry solvents were low, and the large variations between columns packed with the same solvent used would make it hard to exactly predict column performance solely based on slurry solvent and packing procedure. Nevertheless, the probability of getting a column with high PC would be largest for 80 % ACN.
Figure 26 PC at 10 % (A) and 50 % (B) of the peak height for columns packed with different slurry solvents. n≥3 columns were examined (different colors) with 1 ng/µL digested HSA as analyte (1 µL injection volume, n≥3 injection replicates per column) for the main five slurry solvents. All columns (50 µm ID x 150 mm) were in-house packed with 2.6 µm Accucore C18 particles in a polymerized frit column housing. A 30 min 3-15 % solvent B gradient was used with a flow rate of 130 nL/min.

Variations between injection replicates for the same column were found to be 6 % ± 2 % on average for columns packed with 80 % ACN. One of the reasons for the large variation could be peak width variations between different peptides from HSA (as observed in section 4.1). Especially the first eluting peptide (cASLQK) was for some replicates extremely wide (by fronting), which could be a large source of variation. The reason for the large fronting for this early eluting peptide (for some replicates) is not known, but could have been due to movements of particles inside the column, poor refocusing or undiscovered pump instabilities.

In some cases, too little peak smoothing or lack of data points (see Appendix, Figure 45 in section 6.6 for examples) could also have caused some variations because of difficulties measuring the correct peak width. When data points are lacking, the bandwidths are measured at the wrong place/height. The problem with too little peak smoothing were solved by doing measurements after drawing an imagined Gaussian curve along the peak.
The manually drew smoothing could, however, have caused some under/over estimations, although with many replicates be averaged.

Still, variations between intra column replicates did not exceed that between columns (11 %). Hence, all variations were not caused by low chromatographic repeatability. Large random variations for the packing process have been observed [104]. Nevertheless, the packing process itself could also suffer from some gross errors, such as large particle size distribution leading to segregation [100], small pressure differences because the packing process is consuming nitrogen gas from the pressurized tank, differences in depressurization time, or small differences in slurry-concentration due to evaporation (affected by room temperature).

Comparison of packing approaches

No significant difference was observed after visual inspection at neither 10 % nor 50 % of the peak height when the two packing approaches (heat and magnetic stirring) were compared for columns packed with 80 % ACN (Figure 27). Higher column efficiency (i.e. PC) was expected for higher temperatures [88], because of lower viscosity and a higher degree of solvent evaporation prior to pressurization in the pressure bombs (higher total slurry concentrations) at higher temperatures. Higher slurry solvent concentration is a significant factor for increased separation efficiency due to less size segregation inside the column [83, 100].

![Figure 27 Peak capacity for columns packed with magnetic stirring (n=5 columns) and the heat-packing approach (n=3 columns). PC was measured at 10 % (triangles) and 50 % (circles) of the peak height for columns packed with 80 % ACN. The data for magnetic stirring is the same as in Figure 26). The lines show the average PC, and “X” was considered an outlier according to Grubbs test. Other chromatographic conditions were as described in Figure 26, with n≥3 injections of peptides from digested HSA per column.](image-url)
The reason for similar PC for columns packed at room temperature and 65 °C could be lack of stirring with the heat-packing approach. Lack of stirring would give a faster sedimentation rate, which would decrease the slurry concentration accessible to the packing capillary. Stirring during packing with heat was, however, not found practical with the set-up used in the present study. Due to the similar PC and ease of packing with the magnetic stirring approach, magnetic stirring was used for further investigations.

Other sources of variation between columns

The timeframe from sonication to pressurization could also be a source of variation between columns. This timeframe was usually varying because it was done manually (human errors), thus impact the sedimentation level. Treadway et al. [104] found that longer time from sedimentation to packing increased the separation efficiency due to more aggregation. However, this effect is probably less crucial in the present study because only one column was packed with the same slurry, unlike in the study of Treadway et al. where all columns were packed with the same slurry (and with a different set-up).

At the same time, Capriotti et al. [87] proposed increased size segregation/uneven distribution of particles with increased sedimentation (probably due to decreased slurry concentration), which would decrease the column efficiency. On the other hand, they were using totally porous particles. According to other studies, the effect of high slurry concentration would be less for solid-core particles and for d_0/d_p ratios ≤ 25 [100]. Since both solid-core particles and a d_0/d_p ratio of 19 were used in the present study, the low repeatability between columns was probably not only due to slurry concentration differences.

Other sources of variation were probably frit length, cutting and coupling to the LC-MS system. How square the cut is, at the column inlet and frit, and how well connections to the Tee union and emitter are made, would all impact the band broadening and the repeatability. Both column and frit length were difficult to cut to the exact same length (low repeatability) with the polymerized frit used. Longer frits were expected to give rise to larger band broadening because the frit does not give any retention. A stationary phase could have been cross-linked to the polymerized frit, and this is commonly done in the production of silica monolithic columns [86]. However, this would have required additional steps reducing simplicity of the present method. Nevertheless, investigation of frit with stationary phase could be performed in further studies to possibly improve PC.
Neat solvent slurries vs binary slurries

Neat solvent slurries have in the literature been showed to give better performing columns than binary solvents (such as 80 % organic solvent in water) [83]. Because of this, the performance of columns packed with 100 % ACN [94] (n=3 columns) and 100 % MeOH [83] (n=3 columns) was compared to columns packed with 80 % ACN. However, in this study, 80 % ACN still gave the highest peak capacities (Figure 28).

100 % MeOH gave columns with a mean PC of 75 ± 16 at 10 % of the peak height, which was equal to that of both 80 % MeOH and 100 % ACN. Hence, none of the neat solvent slurries produced columns with significantly higher PC than 80 % ACN.

On the other hand, pump instabilities (problems with the flow of solvent B) were observed in the investigation of two of the three columns packed with 100 % ACN. Both columns were excluded in the calculation of average PC in Figure 28 because of much lower efficiency. However, whether the reason is the pump instabilities or random variation is unknown, but a further discussion can be found in Appendix, section 6.4.4. Because all columns (n=3) packed with 100 % MeOH were examined during the time of pump instabilities (could have affected the measurements), 100 % MeOH should preferably be examined further.

Nevertheless, because no significant improvement was found for neither 100 % ACN nor 100 % MeOH compared to 80 % ACN, and a lower content of organic solvent would be both
greener (more environmentally friendly) and cheaper, 80 % ACN was in the present study selected as the better choice (and used for further comparisons).

Frits and New Objective column housings

Shorter and possibly more repeatable frits compared to the polymerized frit could be made by a sintering process, because such frit lengths would be easier to control. To reveal the impact of the frit, columns prepared with polymerized and sintered frit were compared, including the New Objective column housings in the comparison. All columns were packed with magnetic stirring and 80 % ACN as slurry solvent.

No significant difference was observed between the New Objective column housings with integrated emitter (with frit) and columns with polymerized frit, indicating low extra column band broadening in the emitter and associated connections (Figure 29). On the other hand, the PC was significantly lower for the sintered frit. At 10 % of the peak height, the PC for the sintered frit was 48 % lower. The reason was most likely because the polyimide layer, which makes the column mechanically stronger, was removed during sintering, making the OD of the frit end slightly smaller. Hence, the frit did not fit perfectly in the sleeve connecting the column to the emitter, and this could have caused dead volume.

Because New Objective column housings are more expensive than fused silica capillaries, fused silica capillaries were considered the best option and selected for further investigations.
In-house packed nanoLC columns vs a commercial nanoLC column

For the comparison of in-house packed nanoLC columns and a commercial column, the best performing column packed with the optimal packing procedure was selected (80 % ACN packed with magnetic stirring and polymerized frit).

Figure 30 shows a significant higher PC for the commercial column. The commercial column had a PC of 136 ± 5 (315 ± 10) compared to the best in-house packed column with a PC of 102 ± 5 (225 ± 23) at 10 % (50 %) of the peak height. This implies a 33 % (40 %) increase in PC for the commercial column at 10 % (50 %) of the peak height. Chromatograms of the seven first eluting peptides (among the selected peptides) for both columns can also be seen in Figure 31. Note that the different flow rate of the columns (due to different ID) probably has caused the large differences in retention time.

The difference in PC could be due to several reasons. With solid-core particles, it is harder to pack narrower columns with good efficiency (demonstrated for 4.6 mm ID to 2.1 mm ID columns in [129]). A recommended flow rate (probably optimized by the manufacturer) was also provided for the commercial column, while only a theoretical optimal flow was used for the in-house packed columns. For further studies of the in-house packed column, the flow rate could be optimized.

Some band broadening in the in-house packed column chromatogram could be as this column have has smaller ID, and the same amount of digested HSA was applied.
The commercial column had probably also been packed with higher pressures. According to literature, columns should be packed with higher pressures than the expected operational pressure [80, 98]. This was not possible with the packing set-up used in this study. Packing with an LC pump instead of packing bombs was also attempted, but was unsuccessful because a suitable slurry reservoir was not available for the very narrow ID of the capillaries. According to Meyer et. al, downwards packing was more efficient than upwards [110], which was used in this study.

A constant packing rate and slow depressurization have also been observed to be important for achieving repeatable columns with high efficiencies [80, 83]. To obtain a constant packing rate, the pressure during packing would have to be increased concurrently with the buildup of the packed bed. While already operating at the highest possible packing pressure (200 bar), increasing the pressure to several thousand, as used by others [83, 104], was not possible. Slow depressurization was also challenging with the packing set-up used in the present study due to its construction. On the other hand, relatively low pressures and fast depressurization make the set-up used in the present study quick and simple.

Figure 31 EIC of seven of the eight selected peptides for an in-house packed column with 80 % ACN (above) and the commercial column (bottom). One injection replicate from each column is shown. Chromatographic conditions and the same extracted signals as in Figure 25.
4.2.4 Retention time repeatability

Retention time is an important parameter for identification of compounds in a chromatographic analysis, especially when using low selectivity detectors (e.g. low mass resolving power MS). Columns giving repeatable retention times are therefore a necessity for applications in e.g. clinical medicine. Hence, a goal was to pack columns providing high retention time repeatability between injection replicates (intra-column), and between columns (inter-column). C₁₈ was revisited for comparison with C₁₈ in nanoLC format, but both column performance and retention times were similar (see Appendix, section 6.4.5). Hence, only C₁₈ was investigated further.

The average retention time RSD% for the same seven peptides from tryptic digest of HSA as in Figure 25 (section 4.2.3, the last eluting peptide was excluded because of identification issues for some injection replicates) was investigated for columns prepared with the five main slurry solvents (Figure 32) and the commercial nanoLC column. The same chromatographic conditions as for the PC measurements (section 4.2.3) were used (flow rate of 300 nL/min instead of 130 nL/min for the commercial nanoLC column). Because of the known pump instabilities during the investigation of columns packed with neat solvent slurries, these columns were not included in this examination.
Most columns packed with 80 % ACN did not give significant higher retention time RSD% than the commercial nanoLC column. Figure 32 shows the average intra-column RSD% (for the seven peptides combined) for the main slurry solvents (see Appendix, Table 21 in section 6.5.2 for example of calculation). According to Figure 32, this variation was usually less than 3 % for all columns regardless of slurry when outliers were excluded. 80 % ACN gave columns with one of the lowest intra-column median values, but had the largest inter-column variance. Especially one column had extremely low repeatability (up to 4.6 % retention time RSD% for one of the peptides), which correlates to the high PC RSD% for the same column. This observation only indicated a poorly packed column (probably loosely packed), which would be expected to happen occasionally according to previous observations of PC in the present thesis.

On the other hand, the two columns with highest average retention time RSD% were also the first two columns investigated. Hence, unknown (until time of writing) system instabilities could possibly also have occurred. Figure 33 is depicting retention time RSD% for all columns packed with 80 % ACN (see Appendix, Table 21 in section 6.5.2 for example of calculation). The two columns with the highest retention time variance (as also seen in Figure 32) are marked with “x”, because the average intra-column RSD% (for the seven peptides combined) was indicated to be significantly different to the others according to a t-test.

Figure 33 Retention time variation (RSD%) for all columns packed with 80 % ACN compared to a commercial nanoLC column. All points (circles and “X”) in the figure indicate the average RSD% for one peptide from digested HSA. Columns 1-5 were packed with 80 % ACN as slurry solvent (with magnetic stirring in polymerized frit column housings). The black lines show the average intra-column RSD% (for the seven peptides combined). Chromatographic conditions were as described in Figure 26, except for the commercial column (75 µm ID x 150 mm) which was examined with a flow rate of 300 nL/min.
Still, these columns should be presented to show that all in-house packed columns are not perfect. However, when excluding these two columns, the retention time RSD% was at the same level as the commercial nanoLC column. A chromatogram showing retention time stability for the seven of the eight selected peptides chromatographed on column 5 packed with 80 % ACN was given as example in Figure 25 (section 4.2.3). In conclusion, higher variances do occur occasionally, but it is not clear whether this is due to system instabilities or poorly packed columns.

The overall inter-column variation for columns packed with 80 % ACN (C18) is for the seven peptides showed with “x” in Appendix (Figure 42 in section 6.4.5). Some key-numbers are also given in Appendix (Table 22 in section 6.5.2), showing that the retention time between these columns varied with about 1.5 min ± 0.4 min (10 %) on average, compared to ≤ 3 % (mostly) between injection replicates within a column. Figure 34 presents the retention times for seven of the selected peptides for columns 3-5 mentioned in Figure 33 as an example.

![Figure 34 Extracted ion chromatograms (EIC) of seven of the eight selected peptides for three columns packed with 80 % ACN (inter-column repeatability). The chromatograms are showing one replicate for each of the three columns (50 µm ID x 150 mm, 2.6 µm Accucore particles) packed with 80 % ACN (with magnetic stirring in polymerized frit column housings) with the lowest intra-column retention time variation (column 3-5 in Figure 33). Chromatographic conditions as Figure 25. Extracted signals from m/z values were also the same as in Figure 25.](image-url)
A higher inter-column retention time variation compared to intra-column variation was expected because of previous observations (and literature) showing difficulties producing identical columns even with the same procedure. Because the columns were cut by hand after measurements by a ruler, the columns were 150 mm ± 2 mm. Hence, small differences in column lengths (maximum 4 mm) could also have been an important factor for inter-column retention time RSD%. Nevertheless, high RSD% implies that the retention time of the analyte(s) needs to be verified if the column is changed for proteomic studies and other clinical approaches. Unfortunately, whether commercial nanoLC columns suffer from the same retention time shifts is not known.

*To summarize, the intra-column retention time variation was for most nanoLC columns packed with 80 % ACN comparable to the commercial nanoLC column, but larger variations were found between columns.*

### 4.2.5 Application

The in-house packed nanoLC columns packed with 80 % ACN (magnetic stirring and polymerized frit) was successfully used by Kristina Erikstad Sæterdal [128] with a 50 µm ID x 50 mm silica based C₈ monolithic on-line SPE column made by Tone Smetop [130] to identify CYP27a1, which is an important enzyme involved in the oxidation of cholesterol to 27-hydroxycholesterol in the human body and a possible biomarker for breast cancer [131, 132]. CYP27a1 was extracted from grown MDA-MB-231 cells, which originated from a patient with an estrogen receptor negative (ER-) breast cancer type.

For the detection, signature peptides (peptide specific for a given protein) from tryptic digest of CYP27a1 from cell lysate was used. One of these signature peptides (SIPEDTVTFVR) is shown in Figure 35 (three MS/MS transitions) [128], and show very narrow peaks (≤25 seconds at the base line) with excellent peak shape (relatively symmetrical peaks).
Figure 35 EIC of the signature peptide SIPEDTVTFVR from trypsinated CYP27a1 (top) with mass spectrum of fragment transitions (bottom). The EIC show n=3 injection replicates of the same peptide with transitions marked with stars in the mass spectrum (m/z = 632.34→722.42, 632.34→621.37 and 632.34→522.30). The identification was performed with a 50 µm ID x 150 mm in-house packed analytical column with 2.6 µm C18 Accucore particles (packed with 80 % ACN and magnetic stirring in a polymerized frit column housing) and a 50 µm ID x 50 mm silica based C8 monolithic online SPE column. A gradient of 3-36 % solvent B in 30 min with a flow rate of 130 nL/min was employed. The figure was reproduced with permission from [128].
5 Conclusions

In the present study, a simple and inexpensive slurry packing procedure for nanoLC columns of 50 µm ID with 2.6 µm Accucore particles was developed. Due to similar performance and retention times of the C<sub>18</sub> and C<sub>30</sub> stationary phases, the more common C<sub>18</sub> was the main focus. Both New Objective column housings with integrated frit (no need for emitter) and standard fused silica capillaries with in-house made frits were investigated, but no differences were observed. Hence, the band broadening in the emitter was low, and the fused silica capillaries were chosen because of lower cost. Even though polymerized frits requires a polymerization reaction taking several hours (compared to a sintered frit taking approximately 15 min in total), the polymerized frit was much less fragile, easier to make and gave better performance, and was hence chosen.

Among the different packing procedures, heat and magnetic stirring, magnetic stirring was selected based on being simpler, providing faster packing rates for all packing solvents and at least equally good column performance. With magnetic stirring, all slurry solvents provided ≥ 15 cm columns (with Accucore particles) at low packing pressures (≤ 200 bar) compared to literature. Regarding slurry solvent, 80 % ACN gave the fastest packing rate and produced columns with the highest PC.

Despite optimizations, the best in-house packed column (packed with 80 % ACN) had approximately 30 % less PC than the commercial column. However, stable retention times are an equally important feature for identification purposes. Most of the in-house packed columns had equal retention time repeatability to that of the commercial column, indicating a stable packed bed even though lower packing pressure than operational pressure was used.

Due to the (usually) high retention time repeatability, low cost, and simplicity of the packing method developed in the present study, the lower PC than for the commercial column was considered less important. Similar packing cells to the in-house packing bombs used in the present study are also commercially available. Hence, this packing method could easily be implemented in other research laboratories.
5.1 Future work

Investigations of the neat slurry solvents and other slurry solvents could also be performed to hopefully increase the PC further. For further development of the packing method described in the present thesis, the long term stability and maximum number of injections on the columns should be examined. Exploiting this method for other particles than Accucore solid-core particles (e.g. with totally porous particles to investigate differences as indicated by other studies) would also be a natural extension of the present work. In addition, shorter columns with smaller particles could also be investigated to decrease the analysis time in nanoLC-MS proteomics.
References


128. Sæterdal, K.E., *Determination of CYP27a1 in biological samples using nano liquid chromatography mass spectrometry, master thesis*, in Department of Chemistry. (2016), University of Oslo: Oslo, Norway.


6 Appendix

6.1 Supplementary equations

6.1.1 Backpressure

The backpressure (P) in a column is given by equation 12 [34].

\[ P = \frac{\kappa \eta L}{d_p^2} \]  \hspace{1cm} \text{Eq. 12}

In equation 12, \( \kappa \) is a constant, \( u \) is the flow rate, \( \eta \) is the viscosity of the mobile phase, \( L \) is the column length and \( d_p \) is the particle size. According to the equation, smaller particle size result in higher backpressures, as do higher flow rates, longer columns and higher viscosity.

6.1.2 Large volume injections

The maximum injection volume without extra band broadening (\( V_{\text{max}} \)) for a retaining compound dissolved in the mobile phase is given by equation 11 [34].

\[ V_{\text{max}} = \frac{0.25d_c^2L}{\sqrt{N}} * (k + 1) \]  \hspace{1cm} \text{Eq. 11}

In equation 11, \( d_c \) is column ID, \( L \) is the column length, \( N \) is the plate number (measure of chromatographic resolution) and \( k \) is retention factor (measure of the retention on the stationary phase). According to the equation, a lower \( d_c \) implies a lower maximum injection volume to avoid extra band broadening.
6.2 Initial experiments

6.2.1 Sedimentation test with Zorbax particles

A simple sedimentation test was performed with Zorbax particles (only available particle type at the time, 17 mg particles/mL solvent) to investigate the effects of temperature (40 °C and 65 °C) and slurry solvent (ACN/water, EtOH/water, MeOH/water and acetone/water (all 80/20, v/v)). Figure 36, with EtOH/water as an example, confirms the longer sedimentation rate at higher temperatures purposed by the literature (clearly more suspended particles at 65 °C).

Figure 36 Zorbax particles (17 mg/mL) suspended in EtOH/water (80/20, v/v) after 5 min in ultrasonic bath and 5 min rest at 40 °C (left) and 65 °C (right).

ACN/water, EtOH/water and MeOH/water (all 80/20, v/v) were all found to suspend the particles for a longer time after sonication for 5 min, and were selected for further study. However, since the optimal slurry solvent highly depends on the type of particles, the slurry solvent has to be optimized for each individual particle type.
6.2.2 Heat-packing with different particle types

2.6 µm Accucore particles (30 mg particles in 1 mL slurry solvent) were the desired particle type in the present study because they were known to give good chromatographic performance [118]. Because only packing with heat was available at the time, the particle’s ability to be packed to >15 cm were investigated for the selected slurry solvents (n=3 columns per solvent) from the sedimentation test with Zorbax particles (aqueous solutions of MeOH, EtOH and ACN). Packing of the Accucore particles were with the optimal slurry solvent compared to other particle types (Zorbax, YMC, Hypersil and Kromasil) to investigate whether other available particles could outperform the Accucoe particles regarding packing rate. The comparison was only based on packing length, and the goal was to obtain as long columns as possible in a reasonable time frame (1 hour).

The longest packing length for the Accucore particles was obtained by using ACN/water. This slurry composition gave an average packing length of 19 cm ± 2 cm, compared to 12 cm ± 1 cm and 10 cm ± 1 cm for MeOH/water and EtOH/water, respectively. Consequently, ACN/water was selected for the comparison of particle types. Regarding particle type, the Accucore particles could be packed with the longest packing length (19 cm ± 2 cm). However, the length obtained was not significantly different from that of the Hypersil and Kromasil particles (see Table 9).

Table 9 Packing length with STD for different particles. Packing was performed using ultrasonic bath (no stirring magnet) for 1 hour with the in-house made bomb packing system. Slurry concentration was 30 mg/mL in ACN/water (80/20, v/v). n=3 columns were packed for all particle types. See Table 19 (section 6.5.1) for more particle information.

<table>
<thead>
<tr>
<th>Particles</th>
<th>Manufacturer</th>
<th>Diameter (µm)</th>
<th>Stationary phase</th>
<th>Packing length after 1 hour (cm)</th>
<th>STD (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accucore</td>
<td>Thermo Scientific</td>
<td>2.6</td>
<td>C_{18}</td>
<td>19</td>
<td>2</td>
</tr>
<tr>
<td>Hypersil</td>
<td>Thermo Scientific</td>
<td>3.0</td>
<td>C_{18}</td>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td>YMC</td>
<td>YMC Co. (Kyoto, Japan)</td>
<td>3.0</td>
<td>C_{30}</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Zorbax</td>
<td>Agilent Technologies</td>
<td>3.5</td>
<td>C_{18}</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>(Waltham, MA, USA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kromasil</td>
<td>Sigma Aldrich</td>
<td>5.0</td>
<td>C_{18}</td>
<td>15</td>
<td>3</td>
</tr>
</tbody>
</table>

No particular trend in packing length based on particle size was revealed. The differences can neither be explained by pore size, endcapping, surface area nor particle shape (Table 9). Because the longest packing length and best repeatability was obtained with the Accucore particles, these were selected for further examinations.
6.2.3 Sedimentation test with Accucore particles

A new sedimentation test was performed with the Accucore particles with a concentration of 30 mg/mL (15 mg particles in 0.5 mL solvent). Slurries with ACN/water, MeOH/water and EtOH/water (all 80/20, v/v), hexane/acetone (67/33, v/v) [87], and isopropanol was compared at room temperature (~22 °C) and 65 °C. All slurries were sonicated for 10 min before the particles were allowed to settle.

Table 23 and Table 24 (both in section 6.5.2) show the sedimentation processes at room temperature and 65 °C, respectively. Generally, the sedimentation rates increased at higher temperatures. At room temperature, particles in the hexane/acetone mixture had the fastest sedimentation rate, where all particles had settled after approximately 15 min. The ACN/water followed with complete sedimentation after approximately 20 min. After 1 hour, all particles in the aqueous MeOH and EtOH mixtures had settled completely, while some particles still were in slurry after 1 hour in isopropanol. Isopropanol therefore seems to have the closest density to the particles (particle densities were not provided by the manufacturer).

At 65 °C, all solvent had evaporated after the sonication for the hexane/acetone slurry. Hence, this slurry was not investigated further in the sedimentation test with heat, and sonication was prior to packing always performed at room temperature for this slurry. All slurries were, in contrast to the test at room temperature, not performed at the same time because the slurries were taken out of the heated bath for pictures. If too many slurries were investigated at the same time, the slurries would be cooled while pictures were taken. Some cooling occurred anyway, and the heated bath quickly dropped to 59 °C when the sonication was turned off. This could have caused some variations and underestimation of the real sedimentation rate at high temperature.

The sedimentation order for the slurries was with heat equal to that at room temperature, but the general sedimentation rate was found to be longer for all slurries with heat. The least difference from room temperature to heat was found for the EtOH/water mixture. This could be due to concentration alternations due to evaporation (as seen in the pictures). No complete sedimentation was observed for any of the slurries until 40 min (ACN/water mixture).
6.3 Supplementary tables of information

Table 10 All amino acids present in HSA (Figure 37) with the one-letter and three-letter code.

<table>
<thead>
<tr>
<th>One-letter code</th>
<th>Three-letter code</th>
<th>Amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>C</td>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>c</td>
<td></td>
<td>Modified cysteine</td>
</tr>
<tr>
<td>D</td>
<td>Asp</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>E</td>
<td>Glu</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>F</td>
<td>Phe</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>G</td>
<td>Gly</td>
<td>Glycine</td>
</tr>
<tr>
<td>H</td>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>I</td>
<td>Ile</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>K</td>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>L</td>
<td>Leu</td>
<td>Leucine</td>
</tr>
<tr>
<td>M</td>
<td>Met</td>
<td>Methionine</td>
</tr>
<tr>
<td>N</td>
<td>Asn</td>
<td>Asparagine</td>
</tr>
<tr>
<td>P</td>
<td>Pro</td>
<td>Proline</td>
</tr>
<tr>
<td>Q</td>
<td>Gln</td>
<td>Glutamine</td>
</tr>
<tr>
<td>R</td>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>S</td>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>T</td>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>V</td>
<td>Val</td>
<td>Valine</td>
</tr>
<tr>
<td>W</td>
<td>Try</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Y</td>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
</tbody>
</table>

Figure 37 Complete amino acid composition of HSA. Selected sequences for the investigation of nanoLC column performance are marked in grey.
6.4 Supplementary experiments and discussions

6.4.1 Packing rate

Regardless of packing approach, the packing rate was generally observed to decrease with time. Hence, a more detailed study of the packing length as function of packing time was performed for one column. Figure 38 shows that a 15 cm column could be packed in less than 25 min with 80 % ACN and magnetic stirring, and that the packing rate decreases until it reaches a plateau after approximately 70-80 min where the packing almost terminates (even though particles were left in the slurry), confirming a previous report when using constant pressures [97]. During packing, a pressure drop will form over the packed bed (backpressure) as the capillary is filled with particles and give resistance to the flow. Hence, the packing process will terminate when the backpressure is equal to the packing pressure used. To cope with this phenomenon and obtain a constant packing rate (beneficial according to some studies [80, 133]), the pressure can be increased slowly during packing (not possible with the set-up used in the present study).

6.4.2 Influence of high pressures on column performance

The influence of high pressures (only obtainable by an LC pump) on nanoLC columns after packing with pressure bombs was investigated for the ability to increase the density of the packed bed, efficiency and retention time repeatability. For this investigation, one of the in-house packed nanoLC columns was firstly packed with a NanoAcquity UPLC pump (Waters) operated close to maximum pressure (650 bar ± 20 bar) after packing with the pressure bomb. Packing with the pump was performed until a stable packed bed (no movement of particles) had formed. Isocratic conditions with ACN/water/FA (80/20/0.1, v/v/v) were employed. The column length was monitored for 7 days (Figure 39).
Indirectly, gathering of clusters (as seen in Results and discussion, section 4.2.2) can be seen as an increase in packing length from start (point A, 9.8 cm) to the maximum after three days (point B, 10 cm) in Figure 39, because clusters were not taken into consideration when the length was measured in this experiment. Thus, the absolute maximum would have occurred immediately after the pressure raise. However, the length was not measured until 3 days. From this point, the packing length decreased to a stable minimum of 9.9 cm after four days due to a denser packing. Because only one replicate was used, the observations were only an indication of how long the packing time with approximately 650 bar must be to obtain a stable packed bed, and hence repeatable columns. The column length was inspected manually with the microscope during pressurization. Hence, depressurization between e.g. injections in a chromatographic analysis was therefore not accounted for. In addition, the effect of high pressure regarding the measured efficiency was not assessed with this experiment.

Figure 39 Packing length (unfocused particles excluded) as a function of packing days with the UPLC pump at 650 bar (± 20 bar). A: packing length of a column (50 µm ID x 150 mm, polymerized frit, magnetic stirring, 80 % EtOH) immediately after packing with the pressure bomb B: Maximum packing length (all clusters had joined the main packing). C: Optimal packing time. At this point the packing length does not change, and the packed bed is stable. Uncertainty in the measuring points is approximately ± 0.05 cm.
According to studies, higher pressures during packing could increase column performance [80, 82]. A nanoLC column packed with Accucore C<sub>18</sub> particles was therefore evaluated regarding PC and retention time repeatability as function of longer packing times (directly after packing with pressure bombs, and up to 15 hours) with the Easy-nLC1000 pump operated at nearly maximum pressure (800 bar ± 20 bar). Seven peptides from digested HSA spread in the gradient window were selected and used for calculations (Table 11).

**Table 11 The seven selected peptides (from trypsinated HSA) for stabilisation experiments only.** The table includes retention order and protonated mass (MH<sup>+</sup>).

<table>
<thead>
<tr>
<th>Order</th>
<th>Peptide sequence</th>
<th>MH&lt;sup&gt;+&lt;/sup&gt; (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TcVADESAENCtDK (t&lt;sub&gt;a&lt;/sub&gt;)</td>
<td>1498.58</td>
</tr>
<tr>
<td>2</td>
<td>ADDKETcFAEEGKK</td>
<td>1627.73</td>
</tr>
<tr>
<td>3</td>
<td>TYETTEK</td>
<td>984.49</td>
</tr>
<tr>
<td>4</td>
<td>LVNEVTEFAK</td>
<td>1149.61</td>
</tr>
<tr>
<td>5</td>
<td>QNCuELFEQLGEYK</td>
<td>829.38</td>
</tr>
<tr>
<td>6</td>
<td>RHPYFYAPELLFFAK</td>
<td>1899.09</td>
</tr>
<tr>
<td>7</td>
<td>VPQVSTPTLVEVSRNLGK (t&lt;sub&gt;j&lt;/sub&gt;)</td>
<td>1924.07</td>
</tr>
</tbody>
</table>

For the present investigation, a “single-column set-up” was used, where the outlet tubing was connected directly to the analytical column with a union, nut and ferrule (no on-line SPE column, and hence no trapping or pre-column equilibration). The linear gradient was set to 3 % to 36 % solvent B in 30 min with a flow rate of 130 nL/min.

When investigating PC and retention time stability as function of packing time with the LC pump, no significant differences was observed when the pump functionality was optimal (e.g. no air in the tubing). For n≥3 injections for each packing time, the average PC was found to be 50 ± 3 and 88 ± 4 at 10 % and 50 % of the peak height, respectively (Figure 40). The PC repeatability was observed to be somewhat higher when the column was packed at least 1 hour with the LC pump.
Figure 40 Average PC (for peptides from tryptic digest of HSA) chromatographed on an in-house packed nanoLC column with packing times (with 800 bar +/- 20 bar) ranging from 0 to 15 hours. PC was measured at 10 % (PC 10) and 50 % (PC 50) of the peak height. The P-values given indicate no significant difference between the packing times. The error bars show the PC STD (n=3 injections of 1 µL (1 ng/µL concentration) for all packing times). One column (50 µm ID x ~150 mm (+ 200 mm capillary) was investigated. The column was packed with magnetic stirring and 80 % ACN as slurry solvent in polymerized frit column housing. A gradient of 3-36 % solvent B in 30 min at a flow rate of 130 nL/min were employed for all packing times.

It should be noted that the PCs in this experiment were somewhat underestimated due to extra column band broadening from the empty capillary section (25 cm) in front of the packed bed. This extra capillary length was needed during packing to prevent particles from being lost (dragged out), but this part was removed after compression with a LC pump (column washing) in further experiments.

Due to ease and timesaving (and no difference in PC when packing with higher pressures), only a washing step with 80 % ACN (to remove possible contaminants and solvent from packing) with higher pressures (350-500 bar) than the expected operational pressure (300-450 bar dependent on column), was included after packing with pressure bombs.
6.4.3 Stability of the packed bed

In Results and discussion (section 4.2.2, “Stability of the packed bed”), an issue with particle loss from the packed bed after depressurization with the LC pump was observed (i.e. after an injection). After the column was cut to the required length and used for analysis, about 1-2 mm of particles was observed lost from the column inlet (end with no frit) when the column was disconnected (seen in the microscope). Because the pump lowers the pressure prior to a new injection, this pressure drop was expected to be the explanation. Hence, the influence of higher packing pressures than expected operational pressure was investigated to hopefully solve the problem by creating a denser particle bed. An in-house packed nanoLC column (cut until the packed bed) was connected to the LC pump and the pressure was raised to 700 bar, before the column pressure was lowered back to atmospheric pressure prior to any investigations. However, after ~1 mm of particles was still lost from the column inlet.

To solve the problem, a frit in both ends of the column, or a filter could have been used. However, nanoLC columns require very small filters to avoid extra band broadening, and none of the frit methods can be used on both ends of the capillary. A sintered frit (using a torch) cannot be used because the capillary would get too brittle to be used in connections (the polyimide layer would have been removed). On the other hand, the polymerized frit (method 2), which does not affect the polyimide coating and preserves the mechanical strength of the capillary, uses temperatures that exceed the maximum temperature tolerance of the particles used (70 °C).

However, the columns were in the present study used with the obtained method without further alternations because particle loss was mostly observed only the first time the columns were used.

6.4.4 Comparison of neat slurry solvents and binary solvents

In Results and discussion (section 4.2.3, “Neat solvent slurries vs binary slurries”), pump instabilities (solvent B valve) were observed for two out of three columns packed with 100 % ACN (and possibly all columns packed with 100 % MeOH). These instabilities were probably the reason for the split peak observed for the first eluting peptide (cASLQK) for these two columns (Figure 41).
The last eluting peptide was not identified for all columns, so for a fair comparison of the three columns packed with 100 % ACN, calculations of peak capacities solely based on peptides 2 to 7 (instead of peptides 1 and 8) were made (Table 12). Still the PC of columns influenced by instabilities was much lower (more tailing) than the PC calculated for the third column not affected by pump instabilities. Hence, these two columns were excluded in the presented average PC for 100 % ACN in Results and discussion, Figure 28 in section 4.2.3.

**Table 12 PC (at 10 % and 50 % of the peak height) for columns packed with 100 % ACN.** All columns were packed with magnetic stirring in polymerized frit column housings (50 µm ID x 150 mm, 2.6 µm Accucore particles). n≥3 injections of peptides from digested HSA were chromatographed per column. Retention times of peptide number 2 and 7 were used in the PC calculations (instead of peptide number 1 and 8). Chromatographic conditions as described in Figure 26.

<table>
<thead>
<tr>
<th>Column number</th>
<th>Pump instability</th>
<th>PC 10 % of the peak height (peptides 1 and 8 excluded)</th>
<th>PC at 50 % of the peak height (peptides 1 and 8 excluded)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Yes</td>
<td>29 ± 2</td>
<td>80 ± 7</td>
</tr>
<tr>
<td>2</td>
<td>Yes</td>
<td>22 ± 9</td>
<td>54 ± 8</td>
</tr>
<tr>
<td>3</td>
<td>No</td>
<td>39 ± 7</td>
<td>88 ± 16</td>
</tr>
</tbody>
</table>

Figure 41 EIC of the peptide cASLQK \((m/z = 353.68)\) from digested HSA observed for two of the in-house packed columns with 100 % ACN as slurry solvent. The first, main peak of cASLQK was observed too early (7.6 min) compared to the third column packed with 100 % ACN (~12 min, no pump instabilities), while the much smaller second peak appears at a relatively long retention time. The column was packed with magnetic stirring in polymerized frit column housings. The columns were 50 µm ID x 150 mm with 2.6 µm Accucore particles. Chromatographic conditions as in Results and discussion, Figure 25 in section 4.2.3.
If lower amounts of solvent B were delivered because of valve leakages, longer retention time (and more tailing) would be expected. However, the retention time was only increased for one of the two columns exposed to instabilities (approximately 1 minute longer for all peptides than the column investigated without pump instabilities). Hence, the lower peak capacities were probably due to both pump instabilities and just poorly packed columns, which have previously been observed to occur with other slurry solvents. Because the instabilities could have affected the PC, and because the first peptide was missing for these two columns, only the last column was used in comparisons with columns packed with 80 % ACN. Only one replicate does only give indications, but the PC was anyway lower than for the columns packed with 80 % ACN. The two rejected columns had even lower peak capacities, so the possibility that 100 % ACN would give significantly higher peak capacities if more replicates were investigated was considered low.

It should also be pointed out that because the MS was uncalibrated, the mass tolerance in “Proteome Discover” was increased to 50 ppm for the precursor ion and 2 Da for the fragment ions to be able to find any of the peptides for all three columns. Nevertheless, the PC should not be altered because of this.

All columns packed with 100 % MeOH were also examined during the time of pump instabilities, but the effect was harder to predict because all columns were examined under the same conditions. The indicated possible reduction in PC because of pump instabilities for columns packed could possibly explain why the obtained peak capacities for 100 % MeOH were equal to 80 % MeOH (contradiction to the findings in [83]). Hence, 100 % MeOH could preferably be studied further with all instabilities eliminated before making final conclusions.

*However, because no differences were observed in the present study, 80 % ACN was used further.*

### 6.4.5 Investigation of $C_{30}$ vs $C_{18}$ in nanoLC format

NanoLC columns packed with $C_{30}$ were compared to $C_{18}$ to investigate whether the retention time differences observed previously would increase when going from conventional to nanoLC format. The same particle types (2.6 µm Accucore) as the conventional $C_{18}$ and $C_{30}$ columns were used.
All columns were packed with the optimal conditions found in section 4.2 (packing with 80 % ACN as slurry solvent with magnetic stirring in polymerized frit column housings), and with the same chromatographic conditions as previously used for the investigation of nanoLC columns (in Results and discussion, section 4.2.3). Both column performance (PC) and possible retention time difference were investigated.

For in-house packed nanoLC columns, C$_{30}$ provided high PC and slightly longer retention times. However, neither the performance nor the retention time shifts were considered significantly different from C$_{18}$, confirming observations in Results and discussion, section 4.1. Figure 42 shows the average retention times for seven of the eight tryptic peptides from digested HSA (Experimental, Table 7 in section 3.7.2) for each column packed with C$_{18}$ and C$_{30}$.

![Retention time for different peptides from tryptic digest of HSA separated on C$_{18}$ (n=5 columns) and C$_{30}$ (n=3 columns).](image)

Every point in the figure corresponds to the average retention time of the peptide for one column (n≥3 injections per column). “X” represent columns packed with C$_{18}$ and triangles represent columns packed with C$_{30}$. The lines corresponds to the average retention time of the peptide separated on C$_{18}$ (white line) and C$_{30}$ (black lines). All columns (50 µm ID x 150 mm, 2.6 µm Accucore particles) were packed with magnetic stirring and 80 % ACN as slurry solvent in polymerized frit column housings. Chromatographic conditions as described in Results and discussion, Figure 26 in section 4.2.3 (retention time data for the C$_{18}$ column were also obtained from the same data set as the calculated PC in this figure).
The last eluting peptide was excluded in the examination because the retention times were observed in only two or less replicates for most columns. Hence, statistic calculations could not be made. According to t-tests, an indication of significant difference was only found for cASLQK.

*Hence, retention times with C30 were generally considered not to be significantly different from that of C18. However, there was a general trend of increased retention times on C30 (average increase of 1.6 min ± 0.3 min, see Table 13 for more details).*

Table 13 Retention time comparison for seven of the eight selected peptides from tryptic digest of HSA separated on C18 (n=5 columns) and C30 (n=3 columns) nanoLC columns. All columns (50 µm ID x 150 mm, 2.6 µm Accucore particles) were packed with magnetic stirring and 80% ACN as slurry solvent in polymerized frit column housings. n≥3 injections per column was performed. Chromatographic conditions as described in Results and discussion, Figure 26 in section 4.2.3 (retention time data for the C18 column were also obtained from the same data set as the calculated PC in this figure).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Average $t_R$ C18</th>
<th>Average $t_R$ C30</th>
<th>Increase in $t_R$</th>
<th>Increase in $t_R$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cASLQK</td>
<td>10.9 min</td>
<td>12.8 min</td>
<td>1.9 min</td>
<td>17</td>
</tr>
<tr>
<td>ADDKETcFAEGKK</td>
<td>11.7 min</td>
<td>13.4 min</td>
<td>1.8 min</td>
<td>15</td>
</tr>
<tr>
<td>ADDKETcFAEGK</td>
<td>11.9 min</td>
<td>13.7 min</td>
<td>1.8 min</td>
<td>15</td>
</tr>
<tr>
<td>EccEKPLEK</td>
<td>12.2 min</td>
<td>13.8 min</td>
<td>1.6 min</td>
<td>13</td>
</tr>
<tr>
<td>QTALVELVK</td>
<td>16.1 min</td>
<td>17.9 min</td>
<td>1.7 min</td>
<td>11</td>
</tr>
<tr>
<td>QNcELFEQLGEYK</td>
<td>21.9 min</td>
<td>23.0 min</td>
<td>1.2 min</td>
<td>5</td>
</tr>
<tr>
<td>VFDEFKPLVEEPNILK</td>
<td>26.0 min</td>
<td>27.2 min</td>
<td>1.2 min</td>
<td>4</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td></td>
<td><strong>1.6 min ± 0.3min</strong></td>
<td><strong>11</strong></td>
</tr>
</tbody>
</table>
Regarding PC, Figure 43 shows that all columns packed with C\textsubscript{30} had similar performance as the top range of columns packed with C\textsubscript{18}. The average PC on the C\textsubscript{30} columns were 101, compared to 89 for C\textsubscript{18}.

To summarize, C\textsubscript{30} resulted in well performing columns, but still with similar performance to C\textsubscript{18}. The retention time shift for C\textsubscript{30} compared to C\textsubscript{18} was also smaller than expected. Using C\textsubscript{30} was therefore not believed to give a significant effect regarding neither column efficiency nor retention time. Hence, these columns were not investigated further.

Figure 43 PC at 10 % of the peak height for C\textsubscript{18} (n=5 columns) and C\textsubscript{30} (n=3 columns). All point show the PC of one injection replicate, and the lines show the average PC. All columns were packed with magnetic stirring, 80 % ACN as slurry solvent, and were made with the polymerized frit column housings. Data for C\textsubscript{18} (80 % ACN) and chromatographic conditions were as described in Results and discussion, Figure 26 in section 4.2.3.
6.5 Raw data and calculations (tables)

6.5.1 Investigation of conventional C\textsubscript{18} and C\textsubscript{30}

Table 14 Retention times (flow rate of 0.25 mL/min) for LHRH, bombesin, Leu-Enk and Met-Enk separated on the conventional (2.1 mm ID x 150 mmm) C\textsubscript{30} and C\textsubscript{18} columns with 2.6 µm Accucore particles. n=3 injections (5 µL, 0.1 mg/mL) were performed for both columns, using a gradient of 3-36% solvent B in 30 min. Calculations include average RSD % for all peptides on both C\textsubscript{30} and C\textsubscript{18}, and average difference in retention time (with RSD%) between C\textsubscript{30} and C\textsubscript{18} for all peptides.

<table>
<thead>
<tr>
<th></th>
<th>Standard</th>
<th>Replicate 1</th>
<th>Replicate 2</th>
<th>Replicate 3</th>
<th>Average</th>
<th>STD (min)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C\textsubscript{30}</td>
<td>LHRH</td>
<td>5.03 min</td>
<td>4.78 min</td>
<td>5.03 min</td>
<td>4.9 min</td>
<td>0.1</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>Bombesin</td>
<td>5.20 min</td>
<td>5.17 min</td>
<td>5.20 min</td>
<td>5.19 min</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Leu-Enk</td>
<td>5.39 min</td>
<td>5.45 min</td>
<td>5.39 min</td>
<td>5.46 min</td>
<td>0.07</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>Met-Enk</td>
<td>5.28 min</td>
<td>5.12 min</td>
<td>5.28 min</td>
<td>5.22 min</td>
<td>0.09</td>
<td>1.6</td>
</tr>
<tr>
<td>C\textsubscript{18}</td>
<td>LHRH</td>
<td>4.78 min</td>
<td>4.59 min</td>
<td>4.66 min</td>
<td>4.7 min</td>
<td>0.10</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>Bombesin</td>
<td>4.95 min</td>
<td>4.92 min</td>
<td>4.93 min</td>
<td>4.93 min</td>
<td>0.02</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>Leu-Enk</td>
<td>5.19 min</td>
<td>5.11 min</td>
<td>5.18 min</td>
<td>5.16 min</td>
<td>0.04</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>Met-Enk</td>
<td>4.86 min</td>
<td>4.93 min</td>
<td>4.95 min</td>
<td>4.91 min</td>
<td>0.05</td>
<td>1.0</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.3</td>
</tr>
<tr>
<td>Increase in retention time for C\textsubscript{30} compared to C\textsubscript{18}</td>
<td>LHRH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.4 %</td>
</tr>
<tr>
<td></td>
<td>Bombesin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5.2 %</td>
</tr>
<tr>
<td></td>
<td>Leu-Enk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5.7 %</td>
</tr>
<tr>
<td></td>
<td>Met-Enk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.2 %</td>
</tr>
<tr>
<td>Average difference</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5.4%</td>
</tr>
</tbody>
</table>

Table 15 Retention times (linear flow rate of 0.10 mL/min) for LHRH, bombesin, Leu-Enk and Met-Enk separated on the C\textsubscript{30} and C\textsubscript{18} column. All other conditions were as in Table 14.

<table>
<thead>
<tr>
<th></th>
<th>LHRH</th>
<th>Bombesin</th>
<th>Leu-Enk</th>
<th>Met-Enk</th>
<th>Average</th>
<th>RSD %</th>
</tr>
</thead>
<tbody>
<tr>
<td>C\textsubscript{30}</td>
<td>12.86 min</td>
<td>13.22 min</td>
<td>14.14 min</td>
<td>13.50 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C\textsubscript{18}</td>
<td>11.38 min</td>
<td>12.33 min</td>
<td>12.91 min</td>
<td>12.36 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Difference</td>
<td>13 %</td>
<td>7 %</td>
<td>10 %</td>
<td>9 %</td>
<td>10 %</td>
<td>2 %</td>
</tr>
</tbody>
</table>
Table 16 PC for conventional C\textsubscript{30} and C\textsubscript{18} columns with flow rates of 0.25 mL/min and 0.10 mL/min. All other conditions were as in Table 14.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>0.25 mL/min</th>
<th>0.10 mL/min</th>
<th>0.25 mL/min</th>
<th>0.10 mL/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>24</td>
<td>16</td>
<td>26</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>23</td>
<td>16</td>
<td>27</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>22</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>Average</td>
<td>14</td>
<td>23</td>
<td>15</td>
<td>26</td>
</tr>
<tr>
<td>STD</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>RSD%</td>
<td>12 %</td>
<td>4 %</td>
<td>4 %</td>
<td>6 %</td>
</tr>
</tbody>
</table>

Table 17 Increase in peak width (at 10 \% of the peak height) from the 1 µL to 100 µL injection, and from the 50 µL to 100 µL injection for Leu-Enk, Met-Enk, bombesin and LHRH on C\textsubscript{18} and C\textsubscript{30}. A flow rate of 0.25 mL/min was used. All other conditions were as in Table 14.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Increase from 1 µL to 100 µL C\textsubscript{18}</th>
<th>Increase from 1 µL to 100 µL C\textsubscript{30}</th>
<th>Increase from 50 µL to 100 µL C\textsubscript{18}</th>
<th>Increase from 50 µL to 100 µL C\textsubscript{30}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu-Enk</td>
<td>161 %</td>
<td>136 %</td>
<td>23 %</td>
<td>21 %</td>
</tr>
<tr>
<td>Met-Enk</td>
<td>51 %</td>
<td>56 %</td>
<td>6 %</td>
<td>14 %</td>
</tr>
<tr>
<td>Bombesin</td>
<td>22 %</td>
<td>40 %</td>
<td>11 %</td>
<td>14 %</td>
</tr>
<tr>
<td>LHRH</td>
<td>44 %</td>
<td>42 %</td>
<td>14 %</td>
<td>17 %</td>
</tr>
</tbody>
</table>

Table 18 Increase in asymmetry (at 10 \% of the peak height) from the 1 µL injection to the 100 µL injection, and from the 50 µL injection to the 100 µL injection for Leu-Enk, Met-Enk, bombesin and LHRH with C\textsubscript{18} and C\textsubscript{30}. A flow rate of 0.25 mL/min was used. All other conditions were as in Table 14.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Increase from 1 µL to 100 µL C\textsubscript{18}</th>
<th>Increase from 1 µL to 100 µL C\textsubscript{30}</th>
<th>Increase from 50 µL to 100 µL C\textsubscript{18}</th>
<th>Increase from 50 µL to 100 µL C\textsubscript{30}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu-Enk</td>
<td>145 %</td>
<td>150 %</td>
<td>30 %</td>
<td>28 %</td>
</tr>
<tr>
<td>Met-Enk</td>
<td>33 %</td>
<td>55 %</td>
<td>2 %</td>
<td>23%</td>
</tr>
<tr>
<td>Bombesin</td>
<td>- 42 %*</td>
<td>- 18 %*</td>
<td>- 19 %*</td>
<td>-15 %*</td>
</tr>
<tr>
<td>LHRH</td>
<td>9 %</td>
<td>22 %</td>
<td>6 %</td>
<td>14 %</td>
</tr>
</tbody>
</table>

*Total decrease, but fluctuation between tailing and fronting occurred between injections.

Table 19 Properties of Accucore-, Hypersil-, YMC-, Zorbax, and Kromasil particles.

<table>
<thead>
<tr>
<th></th>
<th>Accucore</th>
<th>Hypersil</th>
<th>YMC</th>
<th>Zorbax</th>
<th>Kromasil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter (µm)</td>
<td>2.6</td>
<td>3</td>
<td>3</td>
<td>3.5</td>
<td>5</td>
</tr>
<tr>
<td>Pore size (Å)</td>
<td>80</td>
<td>120</td>
<td>unknown</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>Stationary phase</td>
<td>C\textsubscript{18}</td>
<td>C\textsubscript{18}</td>
<td>unknown</td>
<td>C\textsubscript{30}</td>
<td>C\textsubscript{18}</td>
</tr>
<tr>
<td>Endcapping</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Porous?</td>
<td>Solid-core</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Shape</td>
<td>Spherical</td>
<td>Spherical</td>
<td>Spherical</td>
<td>Spherical</td>
<td>Spherical</td>
</tr>
</tbody>
</table>
6.5.2 In-house packing of nanoLC columns

Table 20 Calculation of PC for the in-house packed columns at 10 % and 50 % of the peak height. The table exhibits one injection replicate with a packed column with 80 % ACN (magnetic stirring and polymerized frit). Conditions as in Results and discussion, Figure 26 in section 4.2.3.

<table>
<thead>
<tr>
<th>t_R (min)</th>
<th>m/z</th>
<th>Peptide sequence</th>
<th>Peak width (10 %, min)</th>
<th>PC 10</th>
<th>Fwhm (min)</th>
<th>PC 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.54</td>
<td>353.68</td>
<td>cASLQK</td>
<td>0.345</td>
<td>10</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>9.95</td>
<td>543.25</td>
<td>ADDKETcFAEEGKK</td>
<td>0.21</td>
<td>17.5</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>10.27</td>
<td>750.32</td>
<td>ADDKETcFAEEGK</td>
<td>0.19</td>
<td>8.7</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>10.52</td>
<td>653.31</td>
<td>EccEKPLLEK</td>
<td>0.215</td>
<td>8</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>16.82</td>
<td>500.81</td>
<td>QTALVEVK</td>
<td>0.4</td>
<td>1</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>22.55</td>
<td>829.38</td>
<td>QCcELFEQLGKEY</td>
<td>0.385</td>
<td>5</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>26.88</td>
<td>682.37</td>
<td>VFDEFKPLVEEPQNLK</td>
<td>0.28</td>
<td>2</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>36.64</td>
<td>830.77</td>
<td>ALVLIAFQYQLQQePFEDHVK</td>
<td>0.176</td>
<td>6</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>

Average 0.2751 99* 0.1234 220**

* Calculation: \( PC_{10} = \frac{t_R - t_a}{w_{av}} = \frac{36.64 \text{ min} - 9.54 \text{ min}}{0.2751 \text{ min}} = 27.1 \text{ min} = 98.51 = 99 \)

** Calculation: \( PC_{50} = \frac{t_R - t_a}{w_{av}} = \frac{36.64 \text{ min} - 9.54 \text{ min}}{0.1234 \text{ min}} = 27.1 \text{ min} = 219.61 = 220 \)

Table 21 Calculation of intra-column retention time variation for columns packed with 80 % ACN (magnetic stirring and polymerized frit). In the table, one column is used as an example (n=3 injections). Other conditions were as in Results and discussion, Figure 26 in section 4.2.3.

<table>
<thead>
<tr>
<th>Column</th>
<th>5</th>
<th>5</th>
<th>5</th>
<th>5</th>
<th>5</th>
<th>5</th>
<th>5</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence order</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>t_R in min (1)</td>
<td>9.50</td>
<td>9.93</td>
<td>10.25</td>
<td>10.5</td>
<td>16.76</td>
<td>22.38</td>
<td>26.84</td>
<td></td>
</tr>
<tr>
<td>t_R in min (2)</td>
<td>9.54</td>
<td>9.95</td>
<td>10.27</td>
<td>10.52</td>
<td>16.82</td>
<td>22.55</td>
<td>26.88</td>
<td></td>
</tr>
<tr>
<td>t_R in min (3)</td>
<td>9.55</td>
<td>9.97</td>
<td>10.3</td>
<td>10.55</td>
<td>16.73</td>
<td>22.31</td>
<td>26.79</td>
<td></td>
</tr>
<tr>
<td>Average t_R (min)</td>
<td>9.53</td>
<td>9.95</td>
<td>10.27</td>
<td>10.52</td>
<td>16.77</td>
<td>22.41</td>
<td>26.84</td>
<td></td>
</tr>
<tr>
<td>STD (min)</td>
<td>0.026</td>
<td>0.020</td>
<td>0.025</td>
<td>0.025</td>
<td>0.046</td>
<td>0.123</td>
<td>0.045</td>
<td></td>
</tr>
<tr>
<td>RSD%</td>
<td>*<em>0.28</em></td>
<td>0.20*</td>
<td>0.24*</td>
<td>0.24*</td>
<td>0.27*</td>
<td>0.55*</td>
<td>0.17*</td>
<td>0.28**</td>
</tr>
</tbody>
</table>

* Different points in Figure 33.

** Point for the 5th column packed with 80 % ACN in Figure 32.

Table 22 Inter-column RSD% of peptides chromatographed on columns (n=5) packed with 80 % ACN (magnetic stirring and polymerized frit). n≥3 injections per column. Other conditions were as in Results and discussion, Figure 26 in section 4.2.3.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Average t_R C_is</th>
<th>STD</th>
<th>RSD%</th>
</tr>
</thead>
<tbody>
<tr>
<td>cASLQK</td>
<td>10.9 min</td>
<td>1.3 min</td>
<td>12 %</td>
</tr>
<tr>
<td>ADDKETcFAEEGKK</td>
<td>11.7 min</td>
<td>1.7 min</td>
<td>15 %</td>
</tr>
<tr>
<td>ADDKETcFAEEGK</td>
<td>11.9 min</td>
<td>1.7 min</td>
<td>15 %</td>
</tr>
<tr>
<td>EccEKPLLEK</td>
<td>12.2 min</td>
<td>1.9 min</td>
<td>15 %</td>
</tr>
<tr>
<td>QTALVEVK</td>
<td>16.1 min</td>
<td>0.6 min</td>
<td>3 %</td>
</tr>
<tr>
<td>QCcELFEQLGKEY</td>
<td>21.9 min</td>
<td>1.5 min</td>
<td>7 %</td>
</tr>
<tr>
<td>VFDEFKPLVEEPQNLK</td>
<td>26.0 min</td>
<td>1.6 min</td>
<td>6 %</td>
</tr>
<tr>
<td>Average</td>
<td>1.5 min ± 0.4 min</td>
<td></td>
<td>10 %</td>
</tr>
</tbody>
</table>
Table 23 Sedimentation test at room temperature. All slurries consisted of Accucore 2.6 µm particles in a 30 mg/mL slurry concentration. Sonication was performed for 10 min (25 -29 °C) and let to settle for 60 min in room temperature.

<table>
<thead>
<tr>
<th>Time</th>
<th>ACN:H₂O (80/20, v/v)</th>
<th>EtOH:H₂O (80/20, v/v)</th>
<th>MeOH:H₂O (80/20, v/v)</th>
<th>Hexane/acetone (67/33, v/v)</th>
<th>Isopropanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start</td>
<td><img src="start1.png" alt="Image" /></td>
<td><img src="start2.png" alt="Image" /></td>
<td><img src="start3.png" alt="Image" /></td>
<td><img src="start4.png" alt="Image" /></td>
<td><img src="start5.png" alt="Image" /></td>
</tr>
<tr>
<td>5 min</td>
<td><img src="5min1.png" alt="Image" /></td>
<td><img src="5min2.png" alt="Image" /></td>
<td><img src="5min3.png" alt="Image" /></td>
<td><img src="5min4.png" alt="Image" /></td>
<td><img src="5min5.png" alt="Image" /></td>
</tr>
<tr>
<td>15 min</td>
<td><img src="15min1.png" alt="Image" /></td>
<td><img src="15min2.png" alt="Image" /></td>
<td><img src="15min3.png" alt="Image" /></td>
<td><img src="15min4.png" alt="Image" /></td>
<td><img src="15min5.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Table 24 Sedimentation test with heat (65°C). All slurries consisted of Accucore 2.6 µm particles in a 30 mg/mL slurry concentration. Sonication was performed for 10 min (65 °C) and let to settle in the heated ultrasonic bath without sonication for 60 min.

<table>
<thead>
<tr>
<th></th>
<th>ACN:H₂O (80/20, v/v)</th>
<th>EtOH:H₂O (80/20, v/v)</th>
<th>MeOH:H₂O (80/20, v/v)</th>
<th>Hexane/acetone (67/33, v/v)</th>
<th>Isopropanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start</td>
<td>![Start]</td>
<td>![Start]</td>
<td>![Start]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min</td>
<td>![5 min]</td>
<td>![5 min]</td>
<td>![5 min]</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>15 min</td>
<td>![15 min]</td>
<td>![15 min]</td>
<td>![15 min]</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Time (min)</td>
<td>Image 1</td>
<td>Image 2</td>
<td>Image 3</td>
<td>Image 4</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>20 min</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>40 min</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>60 min</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
<td></td>
</tr>
</tbody>
</table>
6.6 Supplementary chromatograms

Figure 44 TIC of 0.1 mg/mL Leu-Enk (m/z = 556) chromatographed on the conventional C\textsubscript{18} column (2.1 mm ID x 150 mm with 2.6 µm Accucore particles). The chromatograms show a visually larger asymmetry at a flow rate of 0.10 mL/min (B) compared to 0.25 mL/min (A). A gradient of 3-36 % B in 30 min was used, and 5 µL was injected.

Sequence: ADDKETcFAEGKK
m/z = 543.2475 Da
RT = 11.45-12.05
Column 3, 80 % ACN

Sequence: QNcELFEQLGEYK
m/z = 829.38074 Da
RT = 19.65-20.47
Column 3, 80 % ACN

Sequence: ADDKETcFAEGKK
m/z = 543.24719 Da
RT = 9.85-10.40
Column 5, 80 % ACN

Figure 45 EIC of a “normal” peak (A), too little smoothing (B) and lack of data points in a peak (C) with a solid arrow marking where 50 % of peak height was measured. m/z = 543.25 (A and C) and m/z = 829.38. Lack of smoothing was solved by drawing an imaginary Gaussian curve along the peak (dotted line in B). Dotted arrow in C shows approximately where the “actual” 50 % of the peak height would have been measured with a satisfying number of data points. Chromatographic conditions as in Results and discussion, Figure 26 in section 4.2.3.
6.7 Standard operation procedure (SOP) for packing of nanoLC columns

1. **Preparing frits** (beneficial to prepare frit for several columns at the same time, and need to be prepared at least 4 hours before packing)
   a. Make solutions of Kasil 1624, Kasil 1, and formamide according to the procedure of the manufacturer (ratio 3:1:1). Normally, a total volume of 10 µL is sufficient for approximately 20 columns. For 10 µL, mix 6 µL Kasil 1624 with 2 µL Kasil 1 before adding 2 µL formamide. Mix well with the pipet. The solution should be clear.
   b. Dip the end of a fused silica capillary (inner diameter of own choosing) into the solution, and keep it in the solution for about 3-5 sec.
   c. Mark the column and put in an oven at 100 °C for at least 4 hours (preferably overnight).
   d. Cut the frit to about 1 mm (as square as possible).

2. **Connecting capillaries to the pressure bombs** (see Figure 46)
   a. Place a vial with washing solvent (100 % ACN) (without the cap) in the platform
   b. Fit the capillary into the pressure bomb “top-cap” with the frit-pointing upwards. Cut a small piece off the capillary end that was passed through the top-cap to prevent clogging. To prevent damage on the capillary while connecting the top-cap to the platform, pull the capillary upwards until only about 0.5 cm of the capillary is seen inside the top-cap.
   c. Connect the top-cap to the platform. This connection must be air-tight (a wrench or similar must be used, *point 1. in the figure*).
   d. Push the capillary carefully downwards until it stops at the bottom of the vial inside the pressure-bomb (suddenly more resistance). From there, pull the capillary a bit upwards from the bottom (about 0.5 cm). The capillary need to stay in the vial content (*point 2. in the figure*).
   e. Connect the nut (*point 3. In the figure*) to the top-cap by using a wrench until air-tight.
   f. Turn the nitrogen gas on by turning the main valve, and open *Valve 2 (Figure 47)* by turning the same way the arrow goes (counter clockwise). Make sure *Valve 1 (Figure 47)* is closed by turning the same way as the arrow (clockwise) until it stops. Make sure fluid is coming out of the frit end.
   g. Flush the column for about 5 min.
h. Turn off the nitrogen (turn Valve 2 clockwise first, then slowly release the pressure by turning Valve 1 SLOWLY counter clockwise.

i. Once all pressure is released, loosen the nut.

j. Pull the column about 1 cm upwards (out from the vial content, but still inside the pressure bomb). Close the nut, and repeat point number 2f to dry the column. Now, no fluid should come out of the frit end after 5 seconds. Make sure nitrogen gas goes through the column by holding a finger over the column frit end.

k. When the column is dry (no fluid is coming out), repeat point number 2h.

l. Pull the column out, and loosen the top-cap.

3. Making slurry (Figure 4)
   a. Weigh out 30 mg particles in an autosampler vial with cap.
   b. Add 1 mL solvent (80 % - 100 % ACN).
   c. Add a small magnet.
   d. Put the cap on, shake, and place in an ultrasonic bath for 10 min.

4. Column packing
   a. Repeat point number 2b.
   b. Shake the sonicated slurry vial and place it into the platform (remove the washing solvent first).
   c. Repeat point number 2b-2f.
   d. Place the pressure bomb platform onto a magnetic stirrer.
   e. Pack the column until 2 cm above wanted column length (some particles are dragged out when releasing the pressure). The packing process can be followed by the use of a microscope (particles makes the inner column black, which can be seen in a microscope with 4 x zoom).
   f. When done, repeat point number 2h-2i. Remove the column. Remember markings containing the flow direction (should be against the frit!).
6.8 Publications

Nano-LC in proteomics: recent advances and approaches

In proteomics, nano-LC is arguably the most common tool for separating peptides/proteins prior to MS. The main advantage of nano-LC is enhanced sensitivity, as compounds enter the MS in more concentrated bands. This is particularly relevant for determining low abundant compounds in limited samples. Nano-LC columns can produce peak capacities of 1000 or more, and very narrow columns can be used to perform proteomics of 1000 cells or less. Also, nano-LC can be coupled with online add-ons such as selective trap columns or enzymatic reactors, for faster and more automated analysis. Nano-LC is today an established tool for research laboratories; but can nano-LC-based systems soon be ready for more routine settings, such as in clinics?

Steven Ray Wilson*, Tore Vehus*, Henriette Sjaanes Berg† & Elsa Lundanes†
Department of Chemistry, University of Oslo, Post Box 1035, Blindern, NO-0315 Oslo, Norway
*Author for correspondence:
Tel.: +47 97070953
stevens@kjemi.uio.no