Investigating sample dissolution and breakthrough on HILIC nano capillary columns with digested peptides

Using a PolyHYDROXYETHYL stationary phase

doors

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Abstract
Hydrophilic interaction liquid chromatography (HILIC) for the separation of polar compounds is a very useful tool. HILIC can be used for the separation of entire proteomes and complex mixtures of polar compounds. Due to a high orthogonality with reverse phase liquid chromatography (RPLC) it is also useful in 2-dimensional liquid chromatography (2DLC). Using nano columns, samples with limited availability are easier to analyse due to a lower required sample concentration and injection volume. However, there are several problems with HILIC, such as long equilibration times and poor solubility of polar compounds in the mobile phase. In this paper, problems with sample dissolution and their solutions were researched. Using water to dissolve peptides has a negative impact on the separation in means of peak area, peak identification and symmetry. Another observed limitation was increased sample breakthrough when increasing water content. A trap-and-elute method was deployed to limit the breakthrough. Trap length is an important factor due to limited loadability on nano-capillary columns.
**Abbreviations Table**

- **HPLC**: High performance liquid chromatography
- **RPLC**: Reversed phase liquid chromatography
- **LC**: Liquid chromatography
- **HILIC**: Hydrophilic interaction liquid chromatography
- **NP**: normal phase
- **IEX**: Ion exchange chromatography
- **ACN**: Acetonitrile
- **IPA**: Isopropyl alcohol
- **THF**: Tetrahydrofuran
- **SCX**: Strong cation exchange
- **SAX**: Strong anion exchange
- **SDS-PAGE**: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
- **PolyA**: PolyHYDROXYETHYL A
- **TFA**: Trifluoroacetic acid
- **ESI-MS**: Electronspray ionization mass spectrometry
- **FA**: Formic acid
- **AF**: Ammonium Formate
- **MP**: mobile phase
Introduction

_Hydrophilic interaction liquid chromatography_

Analysis of polar compounds such as peptides, carbohydrates and drugs by HPLC has become a field of interest in recent years.¹ These polar compounds express limited retention on reverse phase liquid chromatography (RPLC) stationary phases and therefore require a new approach for LC separations. Due to the variety in structural functions a range of stationary phases can be deployed for the separations of these compounds. Solvability issues for polar compounds and the need for a stationary phase which separates these compounds based on their polarity point towards hydrophilic interaction liquid chromatography (HILIC) which deploys aspects of RP, normal phase (NP) and ion exchange chromatography (IEX).²

Using water as a strong eluent in NP on bare silica columns results in a different retention mechanism compared to non-aqueous LC.³ This mechanism is highly influenced by the formation of a water layer on the stationary phase, which causes analytes to partition to and from this water-rich layer. The term HILIC was first adopted in 1990 to replace aqueous NP and distinguish between the separation mechanisms of aqueous and non-aqueous NP. HILIC stationary phases are of polar nature and therefore retain compounds based on their polarity. Whilst water is excluded as much as possible in NP, the initial mobile phase composition in HILIC should contain at least 3 % aqueous buffer solution for the formation of a water layer in combination with organic solvent.⁴ As an organic solvent, ACN is the most widely used. The organic part of the eluents must be miscible with water and the usage of methanol instead of ACN in HILIC has been investigated with bare silica HILIC columns. Using methanol on these columns resulted in several pairs of compounds switching elution order, likely due to interactions of methanol’s hydroxyl groups with the water layer.⁵ Other organic eluents like isopropyl alcohol (IPA) and tetrahydrofuran (THF) also showed less separation power for small polar compounds on diol columns compared to ACN.⁶ Unfortunately, despite having a superior separation behaviour, the solubility of peptides and other polar compounds is very limited in ACN. The aqueous buffer in HILIC is the strong solvent so compounds elute by increase of the water content in the mobile phase.

Another aspect of HILIC is the ability to perform ion exchange chromatography (IEX).¹ This method of LC is based upon the interactions between the analyte and the ionized stationary phase and can be separated into two different types: strong cation exchange (SCX) and strong anion exchange (SAX). These ion interactions play an important role in the separation of peptides and tryptic digests due to the different charges present in these complex molecules. A variety on HILIC using these electrostatic interactions is called electrostatic repulsion liquid chromatography (ERLC).⁷ Using a stationary phase with a charge similar to the analyte, the electrostatic interactions can influence the separation by decreasing the retention time. The repulsion effects can be used to decrease the retention of compounds which are most strongly retained in HILIC. This allows for the use an isocratic flow for separation of complex mixtures.

The water-enriched layer on the stationary phase creates an excess of water in comparison to the bulk mobile phase. Hydrophilic analytes partition into this water layer, resulting in retention of the compounds. However, when modelled the theory of partitioning does not hold up. Evidence points to a multimodal separation mechanism in which hydrogen bonding, electrostatic interactions and adsorption onto the stationary phase also play significant roles.⁵ A large variety of HILIC stationary
phases has been developed, each with different separation power. The polarity and the type of functional groups of these stationary phases affects the strength of the interactions which occur in the mechanism, effecting the thickness of the water layer and the electrostatic interactions for instance.

Possible applications for HILIC
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a common method for the separation of proteins and peptides by electrophoresis in which a voltage is applied on a gel to separate proteins based on their mass. The method uses SDS to remove structure, so the mobility only depends on the length of the peptide. SDS binds to the amino acids in a 1 to 2 ratio, creating a similar mass-to-charge ratio for all peptides. The voltage applied on the gel then separates these compounds by charge, but since SDS binds in a 1 to 2 ratio, the charge is proportional to the mass. The presence of many acidic or basic amino acids in the proteins result in an acceleration or deceleration respectively, giving an error in the estimation of the mass of the separated peptides. To couple SDS-PAGE with MS is a very time-consuming process, in which after running the gel many work-up steps are required before MS analysis can be performed. Complex mixtures of proteins have been separated with 2-dimensional gel electrophoresis (2DE) coupled with MS to provide identification of these mixtures. These two dimensions have different separation power, such as the separation by charge followed by the separation of mass. These two methods provide orthogonality and together have a larger separation power than either one of them alone. Even though advances in automation have increased the throughput, limitations to this type of analysis still exist. These limitations include problems with resolution due to the large range of both charge and mass in complex mixtures, problems with solubility and problems with identifying less abundant proteins.

Shotgun proteomics is the separation of an entire proteome with LC coupled with MS for analysis. Unlike SDS-PAGE, this separation method does not require as many work-up steps and is overall less time consuming. Both in shotgun proteomics and SDS-PAGE the proteins are digested before analysing them with MS. The MS results of these proteins are processed using computer programs to profile the biological systems. Shotgun proteomics can also be performed in 2DLC to provide a better separation. Multidimensional protein identification technology (MuDPIT) is a technique developed for the separation of complex peptide mixtures using 2-dimensional liquid chromatography (2DLC). MuDPIT uses a combination of RP and SCX to separate complex mixtures followed by analysis with MS. Analytes are injected onto the SCX column and flushed off with salt pulses, after which the eluting analytes are separated with RP. Using the SEQUEST algorithm, the MS data can be compared to determine the structure of the peptides. One major issue is the limited orthogonality, since peptides with similar charges cluster in SCX. Highly polar analytes are often poorly separated with RPLC. To improve on the separation, a combination of HILIC and RP can be used to provide higher separation power.

2DLC is based on the orthogonality of the stationary phases to provide a better separation than would be possible with only one-dimensional separation. Two commonly used stationary phases in 2DLC are RP and SCX since the two are orthogonal. The only problem with SCX is that similar charges end up eluting in the same timeframe. Due to the limited amount of options for the charges and the total amount of proteins or digests present in some samples, another stationary phase replacing SCX would be ideal, provided it has a more orthogonal behaviour to RPLC. Using HILIC is
one very suitable option since it has several advantages compared to SCX. RPLC and HILIC are more orthogonal than RPLC and SCX as show in figure 1.

![Figure 1: 2D plots of peptide retention of RP and HILIC versus SCX and RP. SCX and RP are less orthogonal due to the clustering of peptides with a similar charge.]

By replacing SCX with HILIC a more orthogonal separation can be achieved. There are several problems with this replacement since the mobile phases of HILIC and RP are opposites. Two possibilities are the use of a trap-and-elute setup or increasing the column size, and thereby increasing the dilution. This is a large sacrifice since the amount of sample is often limited, reducing the signal intensity strongly.

**Limitations of HILIC**

Using HILIC in chromatography offers many opportunities for improved separation of polar solutes, complex mixtures and in 2D setups. However, several problems with this method still exist, such as sample solubility, long equilibration times and the lack of good retention models due to the complicated separation mechanism.

Long equilibration times are necessary when gradients are used on HILIC stationary phases. These equilibration times can take up to 30 minutes, depending on flowrate and column volume. Certain buffers can increase the equilibration time even further due to significant interactions with the stationary phase.

Modelling problems in HILIC arise from the complexity of the retention mechanism. Due to the large range of interactions between the analyte and the column the creation of a reliable model has yet to be achieved.

The problem of solubility of polar compounds prompts the question on sample dissolution. Whilst pure ACN as a solvent yields the best results, other solvents are required. By combining water and ACN the solvability of the compounds was increased, however, this resulted in increasingly distorted signal, especially in early eluting analytes. Three other protic solvents were also investigated in the
research presented by Ruta et al. IPA, MeOH and EtOH all provided a better resolution, with IPA>EtOH>MeOH.

**Investigating HILIC**

Stationary phase

PolyHYDROXYETHYL A (5 um, 200 A) is a silica based, polymer coated stationary phase containing two diol groups as can be seen in figure 2. This stationary phase is not a true diol phase and is protonated more easily. The second stationary phase used was a Luna HILIC 200 stationary phase which contains cross-linked diol groups and is more resistant to hydrolysis than stationary phases without cross-linked groups as can be seen in figure 3.

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**Figure 2:** The structure of Poly-HYDROXYETHYL A which has a two diol groups.

**Figure 3:** The structure of Luna 200, a stationary phase with a cross-linked diol group.

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Mobile phase modifiers

Mobile phase modifiers can be used to improve signal strength and peak shape. In proteomics, the addition of trifluoroacetic acid (TFA) to the mobile phase provide superior peak shape compared to the most used, formic acid (FA). However, TFA is not compatible with ESI-MS due to repression of signal caused by ion pairing, whilst FA is. The ionic strength of FA is significantly lower, which results in poor separation of peptides. Using an ammonium formate (AF) buffer in combination with FA creates a mobile phase with higher ionic strength. Buffered solutions in chromatography stabilize both the charge of the analyte and the charge of the stationary phase. Stabilizing the charges of the
analytes prevents any unwanted interactions between the stationary phase and the analyte which may make the measurements irreproducible. Both FA and AF are volatile, making them very suitable for analysis with MS since they do not repress the signal strength.

Due to interactions with the stationary phase different organic modifiers can result in different influences on the retention. On many HILIC stationary phases the influence of FA and AF with pH 3 leads to increased peptide identifications and improved the separation significantly. However, TFA has a chaotropic nature on PolyA stationary phases and thereby decreases the retention time. A concentration of 10 mM AF is required to improve the separation.

The limited availability of proteomes requires a more sensitive setup, for which nano (75 um) and micro (200 um) LC are very suitable. The high dilution of samples in analytical size (2.1 mm, 2.4 um ID) LC needs a high sample concentration which cannot always be provided when using UV-detection as the detection method. By decreasing the internal diameter (ID) of a column the sensitivity increases proportionally according to equation 1. Both nano and micro LC systems can provide the signal strength required for the analysis of these complex mixtures. Since nano LC is more sensitive, it is more often used making sample limited proteomics research more accessible.

\[ f \sim \frac{d_1^2}{d_2^2} \]

*Equation 1: The relation between the change in internal diameter \((d_1^2/d_2^2)\) and the sensitivity \(f\). Decreasing the diameter increases the sensitivity in a quadratic manner.*
Experimental

Materials
All experiments regarding the analysis on nano columns were performed with a Thermo Dionex UltiMate3000 RSLC nano pump with Chromleleon, Dionex variable wavelength detector and autosampler (1 ul loop). Data concerning the analytical column was obtained with a Agilent 1100 series Quat Pump, autosampler and Bruker MicroTOF-Q mass spectrometer.

The nano columns used were PolyHYDROXYETHYL A (5 um particle size, 200 A pores from PolyLC) bulk stationary phase loaded into capillary columns (). The analytical column (50 mm x 3 mm ID) was Luna HILIC stationary phase (3 um particle size, 200 A pores).

Acetonitrile (uHPLC and MS-LC grade) were obtained from Biosolve (Valkenswaard). AF (Bioultra grade, >99% purity), HPLC peptide standard mixture and alpha-casein from bovine milk were obtained from Sigma-Aldrich. FA (ACS reagent, >98% purity) was obtained from Honeywell Fluka. Water was purified with a Sartorius Arium 611UV.

10 mM AF buffers were prepared by adjusting the pH of aqueous solutions to 3.0 using FA which were added in 3 v% to MP A and in 50 v% to MP B. Both mobile phases were degassed using a Transsonic Digital S sonicator by Elma.

Packing Columns
The packing of capillary columns was done with a PolyA stationary phase (5 um, 200 A). The stationary phase was first emulsified in ACN before injection into the column. Using high pressure, the stationary phase was loaded onto the capillary column. For the packing of the traps, high pressure N₂ was used. A disadvantage of this method is that the bed length cannot be fully controlled leading to variabilities between traps.

Methods
Two mobile phases, MP A and MP B, were prepared in the ratios mentioned in table 1 and were used for all measurements. All measurement had a minimum equilibration time of 30 minutes and ran a gradient from 100% MPA to 100% MP B. Unless specified, 10 mM AF adjusted to pH 3 with FA was used as the buffer.

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>%Buffer</th>
<th>%ACN</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3</td>
<td>97</td>
</tr>
<tr>
<td>B</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 1: Ratios of organic eluents to buffer. All buffers contained an organic modifier which is 10mM AF at pH 3 unless specified otherwise.

For the measurements regarding the trap and elute methods, the direct injections, blanks and sample dissolution tests the runtime of the gradient was 30 minutes.
Sample dissolution
For the investigation of the effect of sample dissolution a setup was made in which 3 samples with different percentages of water were measured on the same column. The sample were prepared according to table 2. The run was done with a gradient, which ran from 1%B to 99%B in 30 minutes, maintained at 99%B for 5 minutes and equilibrated for 30 minutes at 1%B. All measurements were performed in duplicate. The sample that was used for these measurements was a tryptic digest of bovine casein. The concentration of the dissolved sample was 50 g/L.

<table>
<thead>
<tr>
<th></th>
<th>A (µL)</th>
<th>B (µL)</th>
<th>Amount of sample (µg)</th>
<th>%B</th>
<th>Total volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% water</td>
<td>12.6</td>
<td>1.4</td>
<td>2.5</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>30% water</td>
<td>9.8</td>
<td>4.2</td>
<td>2.5</td>
<td>30</td>
<td>14</td>
</tr>
<tr>
<td>50% water</td>
<td>7</td>
<td>7</td>
<td>2.5</td>
<td>50</td>
<td>14</td>
</tr>
</tbody>
</table>

Table 2: The total volumes and percentages of water for the samples used in the sample dissolution tests. All samples had a concentration of 5 g/L with an injection volume was 0.5 µL.

Mobile phase modifiers
To investigate the effect of mobile phase modifiers on a PolyA stationary phase 3 different mobile phases were prepared. This should give insight on the most suitable mobile phase modifier for this type of stationary phase.

<table>
<thead>
<tr>
<th>Modifier</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formic Acid</td>
<td>0.3 v%</td>
</tr>
<tr>
<td>Ammonium formate, formic acid pH3</td>
<td>10mM, pH3</td>
</tr>
<tr>
<td>Trifluoroacetic acid</td>
<td>0.1 v%</td>
</tr>
</tbody>
</table>

Table 13 Modifier content in the strong eluents of the mobile phases for the investigation of modifier influence.

A tryptic digested bovine casein sample (0.5 g/L, 0.5 µL injection) was injected onto a capillary column (10 cm, 75 um ID) containing PolyA stationary phase. The sample was eluted by use of a gradient from 1%B to 99%B in 30 minutes. Every measurement was performed in duplicate. The mobile phases were prepared according to table 3. The sample was detected by UV at a wavelength of 214 nm.

To prepare the mobile phases, the modifier was added to water in the quantities mentioned in table 3, followed by the sonication of the solution. Mobile phase A was prepared by adding water with modifier to sonicated ACN, creating a total buffer content of 3%. Mobile phase B contained 50% buffer solution and 50% sonicated ACN.
**Traps**

*Figure 4: Schematic of the trap and elute setup. In position A the analyte was loaded onto the trap with a flow of 10 uL/min with mobile phase A. After loading the valves were switched to the detecting position B. The analyte was run with a linear gradient from 1%B to 99%B.*

The traps were tested by loading a tryptic digested bovine casein sample (0.5 g/L, 0.5 uL injection) onto the trap column with a 10 uL/min flowrate. After 2 minutes the valve position was switched from position A to position B (see figure 4 for the configuration), after which a gradient from 1%B to 99%B was run. The sample was detected by UV at a wavelength of 214 nm.

**MS data**

For the acquisition of the MS data three different columns were used. The stationary phases, ID and column length are displayed in table 4.

<table>
<thead>
<tr>
<th>Stationary phase</th>
<th>ID</th>
<th>Column length</th>
<th>Flowrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>PolyA</td>
<td>75 um</td>
<td>15 cm</td>
<td>0.4 uL/min</td>
</tr>
<tr>
<td>Luna</td>
<td>3 mm</td>
<td>50 mm</td>
<td>0.7 mL/min</td>
</tr>
<tr>
<td>Polyamide</td>
<td>75 um</td>
<td>11 cm</td>
<td>0.3 uL/min</td>
</tr>
</tbody>
</table>

*Table 4: Stationary phases, ID, column length and flowrates for use in MS data acquisition.*

The MS data was collected by injecting a tryptic digested bovine casein sample (0.5 g/L, 0.5 uL injection) onto the column. A gradient was run from 0%B to 100%B for 17 and 30 minutes. All measurements were performed in duplicate. The chosen flowrates resulted in a flow velocity of 543 cm/h for the PolyA nano column and a 584 cm/h for the Luna analytical column. Matching the flow velocities of the column is required for creating reproducible LC results.
Results and Discussion

To compare the different chromatograms several late eluting peaks were selected. These peaks were properly separated and used to compare the chromatograms.

Sample dissolution

The blank measurements showed a linear correlation between the t0 peak area and the percentage in water with an $R^2$ value of 0.9995 as can be seen in graph 1. The diluted samples followed a logarithmic pattern with much higher peak areas at t0. To determine the upper limit of this function several extra data points are required.

Graph 1: The peak area at t0 plotted against the percentage of water in sample. The samples for the dissolution tests were prepared according to table 2. The injection volume for all injections was 0.5 uL.

Increasing the water content in casein samples resulted in two mayor changes in the chromatograms. Firstly, the increase of water content in the samples resulted in a larger t0 peak whilst the overall visibility of the peaks decreased. This suggest that a larger amount of breakthrough when a larger volume of water is present in the sample. This increase in breakthrough is supported by evidence provided by measuring blank injections as well. As can be seen in graph 1, the t0 area increases in linear fashion with increasing water content. When compared to the injections containing samples, this similarity was not found. A logarithmic relation between the area of the t0 peaks was found. This makes sense since the total amount of sample is not increased so the peak area should move towards an asymptote.
Graph 2: The comparison of the sample dissolution tests when directly injection onto the column. On the left, the separation with 10% water content in the sample. On the right, the separation with 30% water content in the sample. The signals of the sample containing 50% water were not strong enough to be properly displayed. The most intense peak (#3 in the left image) had an intensity of 40 mAU.

A total of seven peaks were compared in the chromatograms of the sample dissolution measurements. These peaks acted as indicators for the effect of water addition and were compared on area, height and shape. As can be seen in graph 2, several of these peaks co-eluted when injecting with 30% water.

Graph 3: Comparison of the peak symmetry between peaks 1, 5, 6 and 7 of the samples containing 10% and 30% water.
The resolution of the peaks did not change significantly since the retention time of most peaks stayed the same, but the asymmetry did change. The difference was significant between the 30% water and the 10% water sample as can be seen in graph 3. Whilst the peaks of the 10% water sample were highly symmetrical, with exception of peak 5, the peaks obtained when injecting a sample containing 30% water deviated largely. This provides evidence for the decrease of proper chromatography.

The overall separation of the compounds was less than ideal. When injecting the sample directly a vaster array of peaks had been separated, as can be seen in graph 4, but comparing chromatograms made it clear that the peaks coelute in t=10–t=18 min region.

<table>
<thead>
<tr>
<th>Peak No</th>
<th>Retention time (min)</th>
<th>Height (mAU)</th>
<th>Area (mAU)</th>
<th>Resolution</th>
<th>Asymmetry</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22.68</td>
<td>19.74</td>
<td>10.82</td>
<td>2.21</td>
<td>1.13</td>
</tr>
<tr>
<td>2</td>
<td>24.17</td>
<td>16.40</td>
<td>3.85</td>
<td>1.09</td>
<td>n.a.</td>
</tr>
<tr>
<td>3</td>
<td>24.58</td>
<td>32.64</td>
<td>7.58</td>
<td>2.58</td>
<td>n.a.</td>
</tr>
<tr>
<td>4</td>
<td>25.49</td>
<td>0.67</td>
<td>0.13</td>
<td>1.15</td>
<td>1.12</td>
</tr>
<tr>
<td>5</td>
<td>26.11</td>
<td>3.12</td>
<td>2.08</td>
<td>1.82</td>
<td>1.74</td>
</tr>
<tr>
<td>6</td>
<td>27.25</td>
<td>6.98</td>
<td>2.18</td>
<td>2.98</td>
<td>1.02</td>
</tr>
<tr>
<td>7</td>
<td>29.00</td>
<td>1.80</td>
<td>0.65</td>
<td>n.a.</td>
<td>1.081209</td>
</tr>
</tbody>
</table>

*Table 5: The retention time, height, area, resolution and asymmetry of the direct injection with a 10% water sample. The peak numbers correlate to the peak numbers in the left graph 2.*

<table>
<thead>
<tr>
<th>Peak No</th>
<th>Retention time (min)</th>
<th>Height (mAU)</th>
<th>Area (mAU)</th>
<th>Resolution</th>
<th>Asymmetry</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22.66</td>
<td>11.42</td>
<td>12.32</td>
<td>1.35</td>
<td>0.71</td>
</tr>
<tr>
<td>2</td>
<td>24.32</td>
<td>23.79</td>
<td>12.64</td>
<td>2.03</td>
<td>1.22</td>
</tr>
<tr>
<td>3</td>
<td>25.78</td>
<td>3.32</td>
<td>1.49</td>
<td>1.18</td>
<td>0.73</td>
</tr>
<tr>
<td>4</td>
<td>26.54</td>
<td>14.84</td>
<td>6.22</td>
<td>1.82</td>
<td>1.23</td>
</tr>
<tr>
<td>5</td>
<td>27.96</td>
<td>12.02</td>
<td>4.75</td>
<td>n.a.</td>
<td>1.37</td>
</tr>
</tbody>
</table>

*Table 6: The retention time, height, area, resolution and asymmetry of the direct injection with a 30% water sample. The peak numbers correlate to the peak numbers in the left graph 2 whilst being represented by the right chromatogram in graph 2.*

<table>
<thead>
<tr>
<th>Peak No</th>
<th>Retention time (min)</th>
<th>Height (mAU)</th>
<th>Area (mAU)</th>
<th>Resolution</th>
<th>Asymmetry</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22.96</td>
<td>2.21</td>
<td>1.22</td>
<td>1.96</td>
<td>1.10</td>
</tr>
<tr>
<td>2</td>
<td>24.25</td>
<td>1.39</td>
<td>0.25</td>
<td>1.13</td>
<td>0.87</td>
</tr>
<tr>
<td>3</td>
<td>24.60</td>
<td>5.16</td>
<td>1.07</td>
<td>6.02</td>
<td>1.12</td>
</tr>
<tr>
<td>6</td>
<td>27.29</td>
<td>1.90</td>
<td>0.67</td>
<td>n.a.</td>
<td>1.00</td>
</tr>
</tbody>
</table>

*Table 7: The retention time, height, area, resolution and asymmetry of the direct injection with a 50% water sample. The peak numbers correlate to the peak numbers in the left graph 2.*

Comparing the peak areas of the peaks in the three chromatograms led to important observations. Due to co-elution of several peaks the area for several peaks increased in the 30% water chromatograms. When compared to the 50% water chromatograms, the decrease of area was very
apparent. In this chromatogram the area of every separated peak was lower, with exception of the t0 peak which was caused by breakthrough.

**Traps**

Two traps, as indicated in table 1 were used for the separation of the casein.

<table>
<thead>
<tr>
<th>Stationary phase</th>
<th>Length</th>
<th>ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly Hydroxyethyl A</td>
<td>0.35 cm</td>
<td>50 um</td>
</tr>
<tr>
<td>Poly Hydroxyethyl A</td>
<td>2 cm</td>
<td>50 um</td>
</tr>
</tbody>
</table>

*Table 8: precolumn/traps which were used.*

The measurements were performed in duplicates, with and without column and with and without trap. This should give a good indication on the difference in separation power between the different lengths and the different stationary phases. The measurements were repeated with two different mobile phases; one containing 0.1 v% TFA and one containing 0.3 v% FA. The results of the trap measurement were interpreted by comparing them to the direct injection method.

Two different traps were used for the determination of the optimum trap length.

<table>
<thead>
<tr>
<th>No.</th>
<th>Retention time (min)</th>
<th>Height (mAU)</th>
<th>Area (mAU*min)</th>
<th>Resolution</th>
<th>Asymmetry</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21.26</td>
<td>1.45</td>
<td>0.39</td>
<td>3.30</td>
<td>1.49</td>
</tr>
<tr>
<td>2</td>
<td>22.56</td>
<td>13.82</td>
<td>5.64</td>
<td>3.52</td>
<td>1.02</td>
</tr>
<tr>
<td>3</td>
<td>24.02</td>
<td>1.26</td>
<td>0.27</td>
<td>1.18</td>
<td>1.02</td>
</tr>
<tr>
<td>4</td>
<td>24.43</td>
<td>3.65</td>
<td>0.76</td>
<td>4.55</td>
<td>1.27</td>
</tr>
<tr>
<td>5</td>
<td>26.45</td>
<td>1.29</td>
<td>0.43</td>
<td>1.49</td>
<td>1.07</td>
</tr>
<tr>
<td>6</td>
<td>27.26</td>
<td>5.11</td>
<td>1.63</td>
<td>3.57</td>
<td>1.06</td>
</tr>
<tr>
<td>7</td>
<td>29.23</td>
<td>0.60</td>
<td>0.23</td>
<td>n.a.</td>
<td>0.73</td>
</tr>
</tbody>
</table>

*Table 9: 0.35 cm traps with AF/FA mobile phase*

<table>
<thead>
<tr>
<th>No.</th>
<th>Retention time (min)</th>
<th>Height (mAU)</th>
<th>Area (mAU*min)</th>
<th>Resolution</th>
<th>Asymmetry</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22.57</td>
<td>25.35</td>
<td>9.80</td>
<td>3.81</td>
<td>0.90</td>
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<td>24.05</td>
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<td>1.51</td>
<td>1.20</td>
<td>0.97</td>
</tr>
<tr>
<td>3</td>
<td>24.45</td>
<td>6.57</td>
<td>1.39</td>
<td>2.76</td>
<td>1.02</td>
</tr>
<tr>
<td>4</td>
<td>25.58</td>
<td>0.94</td>
<td>0.28</td>
<td>1.55</td>
<td>1.09</td>
</tr>
<tr>
<td>5</td>
<td>26.39</td>
<td>2.20</td>
<td>0.76</td>
<td>1.43</td>
<td>0.97</td>
</tr>
<tr>
<td>6</td>
<td>27.19</td>
<td>11.05</td>
<td>3.79</td>
<td>2.71</td>
<td>1.25</td>
</tr>
<tr>
<td>7</td>
<td>29.15</td>
<td>1.28</td>
<td>0.69</td>
<td>n.a.</td>
<td>1.09</td>
</tr>
</tbody>
</table>

*Table 10: 2 cm traps with AF/FA*

The retention remained identical, indicating that the length difference in the traps did not have any bearing over the overall separation, however, area and height of the peaks were influenced by the increase in trap length. A large increase in both area and height indicate that the performance with the 2 cm trap is better. This effect is likely the result of the breakthrough which was observed in the
direct injection chromatograms. This means that the 0.35 cm trap did not contain enough stationary phase to retain all compounds. To confirm this, two different conditions should be tested. At least one trap length besides 0.35 and 2 cm should be tested to create a pattern. The sample should also be run without any trap present to determine the amount of co-eluting analyte.

The chromatograms had several differences. Whilst using the same sample the symmetry in the second chromatograms improved significantly. Two other mayor differences are the area and peak height which have increased for several peaks in the 2 cm chromatogram. This could be the result of the breakthrough which was observed in the direct injection chromatograms.

The final peaks were very comparable due to mayor similarities in the relative height, area and retention times, whilst earlier eluting peaks between 10 and 18 minutes were not comparable. The 2 cm trap has a very similar peak shape, a cluster of unseparated peaks, in this part of the chromatogram. The 0.35 cm trap showed only a single peak instead of this cluster.

Comparing the traps to direct injection
For the comparison of the direct injection and the trap and elute method the sample concentration should be taken into consideration. The direct injections required high injection concentrations, so there was settled on a concentration of 5 g/L, whilst the sample concentration for the traps was only 1 g/L.

![Graph 4: Comparison of a casein injection on a nano PolyA column (blue) and using a 2 cm trap with a 5 times diluted sample.](image)

The inclusion of the traps resulted in the requirement of a 5 times less dilute sample to achieve a very similar intensity. As can be seen in graph 4, the trap and elute method did not have a t0 peak and the eluting Besides these differences, the mayor peaks later in the chromatogram are very
similar in shape and size. Highly retained compounds are less influenced by the by the separation conditions. Direct injection resulted in breakthrough which is the cause of the comparable peak size.

**Mobile phase modifier**
Unreproducible was obtained with the measurements of mobile phase modifiers. After using 0.1% TFA in the mobile phase the data became irreproducible (Appendix 1). The low pH of this mobile phase might be the cause of this.

**MS data**
Comparing MS data of both the PolyA nano column and the Luna analytical column with the UV measurements led to two significant observations. A lower sample concentration was required for both columns to achieve good signal strength. Still the analytical column required a large amount of sample to achieve acceptable signal strength. Another important observation was the increase in identifiable peptides. Both the analytical column and the PolyA nano column had increased peptide identification compared to the LC-UV.

The casein samples were also separated with a polyamide nano column. The PolyA stationary phase improved on this separation in both the 17 and 30 minutes runtimes. The mayor difference between the obtained signals of both stationary phase is the elution time of some compounds. Many compounds elute later in the run of the polyamide nano column, likely due to electrostatic interactions. These interactions are not present on either the PolyA stationary phase or the Luna stationary phase.
Conclusion
Including traps in the setup improves the signal due to significant loss of sample when injecting an analyte directly. It also focuses the compounds on the trap and facilitates the exchange of injection solvent with the initial mobile phase. Loadability of the traps is a significant issue as can be seen with the lower signal strength obtained with a shorter trap.

The use of TFA is not compatible with the used PolyA stationary phase.

Using UV as an analysis method is limited by since the number of identifiable peaks is limited. This is especially noticeable on the analytical column. Furthermore, large quantities of sample are required for analysis with the analytical column. Using MS instead of UV reduced the amount of sample required for the analysis. Decreasing the column diameter decreases the amount of sample required for the analysis with both UV and MS even more.

Diluting samples with water causes a logarithmic increase of breakthrough of analyte on nano capillary columns. Samples should contain as less water as possible to obtain minimal breakthrough. The peak shape also improves when the water content is decreased.

Outlook
The inclusion of traps in shotgun proteomics could prove a very useful strategy in the separation. By reducing the sample preparation time and the possibility for sample clean-up this method should improve much on the current method of injections. To further the research on trap-and-elute methods a series of experiments should be conducted. At least five data points should be compared, including a measurement without any traps and all should be drawn from the same sample. This should show if there is any loss to breakthrough and if there is a maximum for the trap length. Limiting breakthrough is a significant improvement for measuring samples with limited availability.
Appendix 1: Additional LC data

Graph 1: Injection of tryptic digested bovine casein (10 uL, 5 ug/uL) on an analytical size column (Luna stationary phase, ED, separated with a gradient (0%B to 100%B in 30 minutes). On the Y-axis the absorbance in AU is represented, whilst the x axis represents the retention time.
Graph 2: Chromatogram of separation of casein using a 2 cm trap. Peaks between $t=15$ and $t=18$ co-elute.
Graph 3: Chromatogram of separation of casein using a 0.35 cm trap. A shorter trap results in a lower signal strength. The loading time of the trap was shorter compared to the 2 cm trap. Only one peak appears at t=12. Comparing this chromatogram with the 2 cm trap and the direct injections shows that this peak is a combination of poorly separated peaks.

Graph 4: Direct injection of casein with TFA as mobile phase modifier.
Graph 5: Second injection of casein with TFA as mobile phase modifier. After the use of TFA in the mobile phase no separation yielded reproducible results.

Graph 6: Direct injection with FA as a mobile phase modifier, after using TFA as modifier.

Graph 7: Direct injection of a standard peptide mix on the nano column before using TFA.
Graph 8: Direct injection of a standard peptide mix after using TFA using condition identical to the separation of graph 7.
Appendix 2: Additional MS data

**Graph 1:** Polyamide 17 min gradient on a nano column.

**Graph 2:** Polyamide 30 min gradient on a nano column.
Graph 3: MS data obtained after separation of casein with nano LC on a PolyA capillary column (75 um). The sample was separated by a gradient from 0%B to 100%B in 17 minutes.

Graph 4: MS data obtained after separation of casein with nano LC on a PolyA capillary column (75 um). The sample was separated by a gradient from 0%B to 100%B in 30 minutes.
Graph 5: MS data obtained after separation of tryptic digested casein sample on an analytical size Luna stationary phase column. Injection volume equals 20 uL with a concentration of 0.5 ug/uL. The runtime was 17 minutes.
Graph 6: MS data obtained after separation of tryptic digested casein sample on an analytical size Luna stationary phase column. Injection volume equals 20 uL with a concentration of 0.5 ug/uL. The runtime was 30 minutes.
References

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