Thesis for the Master's degree in chemistry

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Porous layer open tubular columns for protein separations

60 study points

DEPARTMENT OF CHEMISTRY
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1. PREFACE

The work presented in this master thesis was carried out at the Department of chemistry, University of Oslo from January 2008 to November 2009. Prof. Elsa Lundanes, Prof. Tyge Greibrokk and Post Doc. Steven Wilson served as my supervisors.

Early in this study, preliminary experiments were performed to gain knowledge about micro liquid chromatography on particle packed columns before the more challenging chromatography on porous open tubular columns. Some of this work can be found in appendix. A manuscript based on results from this thesis, titled "Separation of intact proteins on porous layer open tubular (PLOT) columns" has been submitted for publication

I would like to thank my supervisors for giving me the opportunity to take a masters degree in organic analytical chemistry, and for giving me an interesting and challenging project. Thank you for the guidance and motivation. Special thanks to Steven Wilson for providing help at any time.

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Furthermore, I would like to thank my fellow students at the group. Thanks to Undis and Ida for motivation and all the interesting discussions. Thanks to Elin and Kristin for pretending to laugh of all my jokes.

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Finally, I would like to thank Nalle Puh and my family for their support.

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Magnus Røgeberg

2. ABBREVATIONS AND DEFINITIONS

2D	2-dimensional
AIBN	Azobisisobutyronitrile
As	Asymmetry
β-A	β-lactoglobulin A
β-B	β-lactoglobulin B
BPC	Base peak chromatogram
CA	Carbonic anhydrase
Cyt C	Cytochrome C
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
DTT	D,L-Dithiothreitol
DVB	Divinylbenzene
EIC	Extracted ion chromatogram
ESI	Electrospray ionisation
FA	Formic Acid
GE	Gel electrophoresis
γ-MAPS	3-(trimethoxysilyl)propyl methacrylate
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
IAM	Iodoacetamide

i.d.	Inner diameter
IR	Infrared radiation
LC	Liquid chromatography
Leu	Leucine
LFD	Large field detector
LOD	Limit of detection
Met	Methionine
MS	Mass spectrometry
Myo	Myoglobin
m/z	Mass to charge ratio
o.d.	Outer diameter
OT	Open Tubular
PEEK	Polyether ether ketone
PLOT	Porous layer open tubular
PSD	Particle size distribution
PS-DVB	Poly(styrene-divinylbenzene)
RP	Reversed phase
SEM	Scanning electron microscopy
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
t_0	Zero retention time
TFA	Trifluoroacetic acid

TOF	Time of flight
t_R	Retention time
Tris	Tris(hydroxymethyl)aminomethane
UHPLC	Ultra-high performance liquid chromatography
UV	Ultraviolet
$w_{0.5}$	Peak width at half peak height
$w_{0.1}$	Peak width at ten percent of the peak height

3. ABSTRACT

Porous Layer Open Tubular (PLOT) poly(styrene-divinylbenzene) columns have been prepared and used for separating intact proteins with gradient elution. The 3 m × 10 µm i.d. columns were easily coupled to standard liquid chromatography-mass spectrometry (LC-MS) instrumentation with commercially available fittings and nanospray interface. Standard proteins separated on PLOT columns appeared as narrow (less than 0.25 minutes wide at half peak height) and symmetrical peaks with good resolution. The within-and between column repeatability retention times (t_R) were below 0.6% and below 2.5% (relative standard deviation, RSD), respectively. The carry-over after injection of 0.5 ng per protein was less than 1.1 %. The column temperature in the range 20 to 60 °C was also evaluated as a separation parameter. The system worked well for separating proteins in milk. The PLOT columns performed well under conditions suitable for on-line protein separation-tryptic digestion. This study has shown that PLOT columns are promising separation tool in top-down proteomics.

4. INTRODUCTION

4.1 Proteomics

The term "proteome" appeared in literature in the mid 1990s, describing the ensemble in a living cell or an organism, related to the genome expressing of these proteins [1]. The proteome is more variable than the genome, and the protein expression varies among cell type, tissue, physiological and environmental conditions. Post translational modifications, alternative splicing, cleavage and break-down products are processes which increase the complexity for proteins after translation [2]. These processes also increase the concentration range from the most abundant to the least abundant protein, also known as the dynamic range, which is known to exceed 10^{10} in plasma [3]. Proteomics can be defined as the systematic analysis of proteins for their identity, quantity and function [4]. Proteomics will give better understanding of disease processes, development of new biomarkers for diagnosis, early detection of diseases, and prediction of new drugs [5].

The largest challenge in proteome analysis is the sample complexity and the large dynamic range. Mass spectrometry (MS) is the most used method for detection in proteomics, but it can only tolerate a certain amount of sample, and the dynamic range is only 10^3 in a single spectrum [3], hence protein overlap should be minimized, by separation for identification and quantification of both the low and high abundant proteins.

The most used approach to separate proteins before MS detection is 2-dimensional gel electrophoresis (2D-GE) [6]. Already inn 1975 O'Farrell described the 2D separation of proteins on a polyacrylamide gel. Proteins were separated according to isoelectric point by isoelectric focusing in the first dimension, and according to molecular mass by sodium dodecyl sulfate electrophoresis in the second dimension (SDS-PAGE) [7]. Even though 2D-GE is the workhorse in proteomics, the trend is moving toward non-gel based separation because of the many limitations; Proteins with high (> 150 kDa) and low (< 10 kDa) molecular masses, extreme pI (especially high pI) and hydrophobic proteins (membrane proteins) are difficult to observe by standard 2D-GE. The linear range is limited to 10^4 and sample handling is done manually, time consuming and difficult to couple on-line with MS [8-10].

4.2 Liquid chromatography in proteomics

A common method for protein analysis is to tryptic digest the proteins before separation and identification by high performance liquid chromatography mass spectrometry (HPLC-MS/MS), an approach referred to as "bottom-up" [11]. Tryptic digestion is a proteolytic reaction which cleaves peptide chains at the carboxyl side of the amino acids lysine or arginine, except when either is followed by proline. This digestion cut the protein into peptides which drastically increases sample complexity. For instance a plasma sample with 30 000 proteins, and if the proteins produce 30 peptides each in average, the digested sample would contain 900 000 peptides. Due to the inherent complexity, there are several disadvantages of the bottom-up approach, one being the high resolving power required, and often a 2-dimensional liquid chromatography (2D-LC) system has to be used [12]. Even though separation of thousands of peptides is achieved in shotgun proteomics, the molecular information about the intact protein is limited; especially for those containing post translational modifications (PMT). These PMTs can involve proteolytic trimming or decoration with more than 100 known chemical groups [13]. The sequence coverage is also generally lower in the bottom-up approach [14]. The sequence coverage simply means the extent to which the entire protein sequence is represented by MS data. E.g. if a tryptic digest of a mixture of a 100 amino acid protein is analyzed and MS data on tryptic peptides corresponding to 60 residues is obtained, then the sequence coverage is 60% [15].

An alternative is the separation and mass spectrometric detection of intact proteins which is called "top-down" proteomics. Figure 1 illustrates both the bottom-up and top-down approaches. Although top-down samples are less complex, the main disadvantages with the top-down approach are limited resolution and recovery, carry-over problems in chromatography and the need of a high-end MS for protein identification [16-18].

Many biomarkers are believed to be low-abundance proteins, and peptides from the low abundance proteins are often co-eluting with other peptides in a bottom-up approach, making identification and quantification difficult. Therefore the top-down approach has great potential in biomarker discovery.

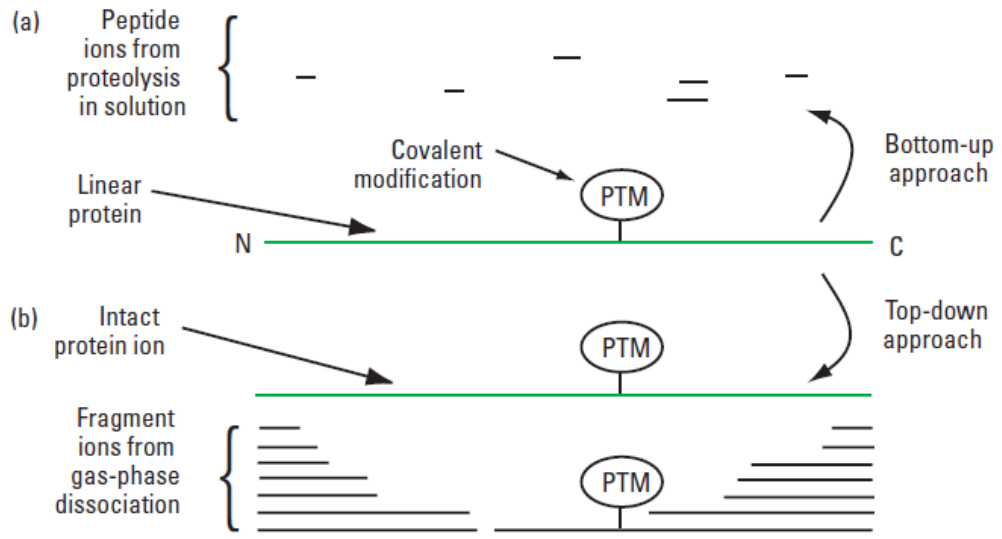

Figure 1. (a) bottom-up and (b) top-down approaches for DNA-predicted protein sequence analysis. The sequence coverage typically 5-70% for bottom-up and 100% for top down. Figure reprinted from [14].

4.3 Downscaling chromatographic systems

Small samples are frequently encountered in proteomics, and the analytes are present in low concentrations hence, it is important to be able to control LC-MS sensitivity parameters. A reduction in the column inner diameter produces a higher sample peak concentration in the detector. The maximum analyte concentration in the column eluate (C_{max}) is given by

$$C_{\max} = \frac{mN^{1/2}}{(2\pi)^{1/2} V_0 (1+k)} \qquad \text{Eq. 1}$$

Where m is the mass of analyte loaded into the column, N is the column efficiency, V_0 is the column dead volume and k is the retention factor. Since V_0 is a function of the inner diameter (i.d.), it can be calculated that the C_{max} ratio for two different i.d. columns would be equal to the ratio of the squares of their i.d. values [19]. Thus, with the same amount of sample injected, decreasing the column i.d. from 500 to 50 µm would result in a theoretical gain in concentration at the detector of one hundred. Several groups have used small i.d.

columns to achieve high sensitivity in proteomics [20-22], in part because electrospray ionization (ESI) MS is concentration sensitive over a wide range of flow rates [23]. Another advantage with low flow is the increased electrospray ionization efficiency because of the formation of smaller droplets (Figure 2) [24]. At sufficiently low flow rates (20 nL/min and lower) and analyte concentrations the ionization efficiency approach 100% and suppression and matrix effects are nearly eliminated [25].

Miniaturizing is essentially reducing the i.d. of a column. However, in order to achieve maximal performance of these columns, the dead volumes before and after the columns are critical. Columns should preferably be coupled directly to the electrospray emitter, either with tubing or a special made "zero dead volume" union [26,27]. This is done both to ensure low dead volumes, but also to prevent torsion during assembly which causes particles that can clog the emitter. Unless phase focusing is utilized, the dead volumes between sample introduction and columns also must be minimized.

The challenge is to develop separations at low nanoliter/min flow rates that can handle typical sample volumes, provide high-efficiency separations and be effectively coupled to ESI-MS.

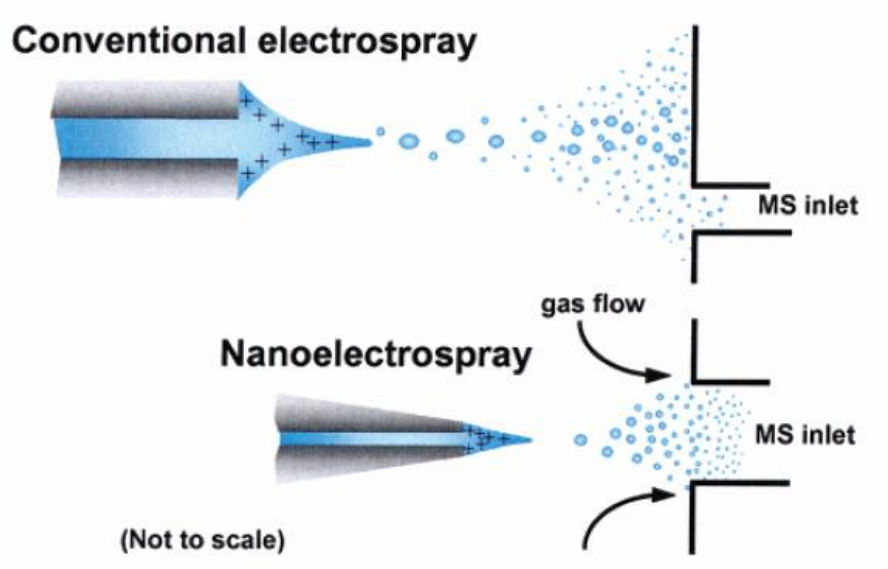

Figure 2. Normal flow rate electrospray (top) and lower flow rate electrospray (bottom). The nanoelectrospray produces smaller droplets and gives a more efficient ion introduction. Figure reprinted from [24].

4.4 Column formats

There are three main column formats used in liquid chromatography; particle packed columns, monolithic columns and open tubular columns. The most common and also the oldest are columns packed with particles. The particles are usually packed inside a steel housing or capillary and held inside by the use of retaining frits which allows liquids to flow through. Monolithic materials consist of a single porous piece of separation media (Figure 3) which is attached to the walls in the columns and do not need frits. The newest format that has been successfully used in LC is open tubular (OT) columns. OT columns resemble the open tubular columns found in gas chromatography (GC) [28,29], but have had a limited use in liquid chromatography because of the slower diffusion in liquids. In order to gain the same efficiencies as good packed columns, LC open tubular columns inner diameter must be 10 µm or less [30].

4.5 High performance liquid chromatography

HPLC with packed columns operated at conventional pressures (<400) has been used to separate both proteins and peptides [16,31]. Some advances in column performance have

been made, such as introduction of larger pore size particles for separation of peptides and proteins, narrower particle size distribution and improved packing procedures [32,33]. Still the problems encountered both in bottom-up (too high sample complexity) and top-down proteomics (limited resolution, recovery and carry-over) demand new column technologies.

4.6 Ultra-high performance liquid chromatography

The most straightforward way to improve the efficiency of a column is to increase the column length (L) or to reduce the plate height (H). For columns packed with particles, the plate height can be reduced by reducing the particle diameter (d_p).

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

Both these ways are limited by the pressure drop over the column. The pressure drop over a packed column is given by:

$$\Delta P = \frac{uL\eta\phi}{d_p^2} \quad \text{Eq. 3}$$

were u is the linear mobile phase velocity, L is the column length, η is the mobile phase viscosity and φ is the column resistance factor. With the same flow, an increase in column length or decrease in particle size by two would give an increase in pressure by two and four respectively. However the optimal linear velocity (μ) is inversely proportional to the particle diameter

$$\mu_{opt} = \frac{3D_m}{d_p} \quad \text{Eq. 4}$$

where D_m is the diffusion coefficient in mobile phase [34]. This implies three points: (1) analysis time will be reduced at optimum flow rates for columns packed with smaller particles, (2) even greater pressure is needed to obtain the optimal flow rate, and with small particles (3) the optimal flow rate for large molecules (low D_m) are lower than for small molecules.

Much work have been done on the separation of peptides with ultra-high performance liquid chromatography (UHPLC) with very high resolution, both with long columns packed with conventional sized particles and columns packed with smaller particles [35-37]. High resolution is also seen for the separation of intact proteins with UHPLC [38]. In addition to the improvements in resolution and analysis time, other unanticipated benefits from operating at ultrahigh pressures can occur. Eschelbach et al. have shown that the carry-over of intact proteins was eliminated for the protein standards (ranging from 13.5-67.0 kDa) at pressures above 1580 bar [39]. The recoveries for ribonuclease A and ovalbumin approached 100% at ultrahigh pressure, versus 50-60% under normal pressure. Recovery improvements for bovine serum albumin were more marginal, indicating that UHPLC might not give quantitative recovery for all proteins.

UHPLC drawbacks are high operating pressures, and the reduction in protein carry-over was seen at a higher pressure than commercially available UHPLC pumps can provide [40-43]. Smaller retaining frits are also required for smaller particles, leading to a higher possibility for column clogging.

4.7 Monolithic materials

The main advantage of monoliths is their high permeability and low mass transfer resistance. Their high permeability is partially obtained by their high porosity, which can reach 80% and is much larger than for the packed columns (having typically 40% porosity). In a simplified view, the analytes are delivered by flow, not by diffusion, leading to their low mass transfer

resistance [44]. This enables highly efficient separations of large molecules. Monolithic columns are commercially available in a vide range of inner diameters, including preperative, analytical and nano size [45-48]

There are two main types of monolithic materials, the organic polymer based, and the inorganic silica based (Figure 3). The silica based monolithic columns were already introduced by Tanaka et al. in 1993 [49]. Since then these columns have been successfully used to separate small molecules and peptides [50,51]. However the organic polymer based monolithic columns have appeared to be better for separation of larger molecules, such as proteins, nucleic acids and synthetic polymers [44]. Therefore the organic monoliths are more interesting for proteomics and especially in the top-down approach.

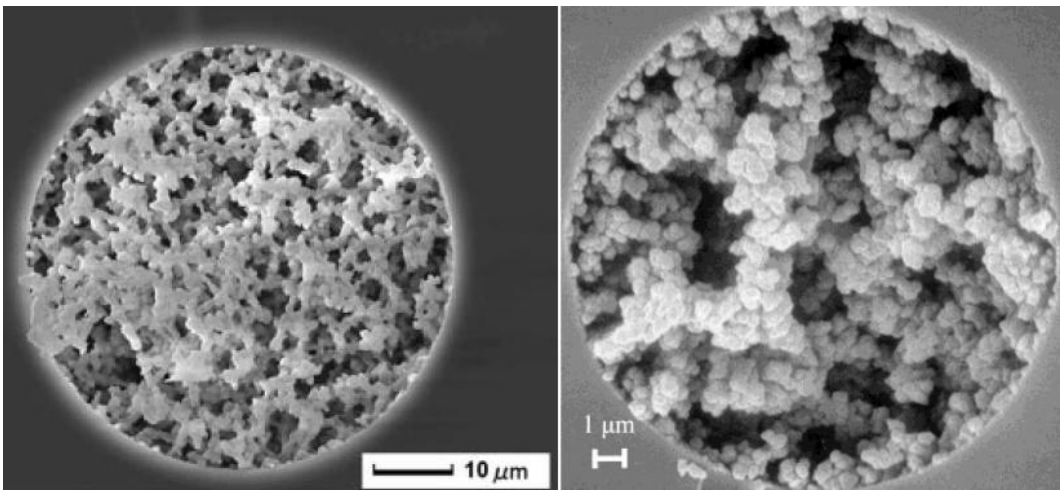

Figure 3. Scanning electron microscopy (SEM) images of an inorganic (left) and organic (right) monolithic column. Reprinted from [52] and [26] respectively.

4.7.1 Properties of organic monoliths

In order to obtain a large surface area, a large number of smaller pores should be incorporated into the polymer. The major part of the surface area comes from the micropores, with size smaller than 2 nm, followed by the mesopores ranging from 2-50 nm. Macropores (>50 nm) contribute very little to the overall surface area, but at essential to allow liquid to flow through the material at a low pressure [53].

The pore size distribution is an important parameter, since linear macromolecules with a molecular mass exceeding 10^4 can not enter the micropores, only the mesopores and the macropores. Because the surface area of the macropores is insignificant, the mesopores constitute the most important part of the entire porous structure for large molecule separations [54].

The chromatographic accessibility also affects the loading capacity of monoliths compared with particle packed columns. The loading capacity is often set to the maximum amount of an analyte that can be loaded into a column with no more than 10% increase in peak width at half peak height. For smaller proteins (<15 000 Da) the loading capacity for poly(styrene-divinylbenzene) (PS-DVB) beads are about ten times larger than for a monolith made from the same monomers. With larger proteins (>50 000 Da) the loading capacities are about the same for both formats [55].

4.8 Preparation of organic monolithic materials

The preparation of organic polymer monolithic columns in fused silica capillaries is usually a 3-step process; pre-treatment, silanization and polymerization.

4.8.1 Pre-treatment

The pre-treatment step is used to prepare the surface inside the fused silica capillary for the silanization (and also function as a washing step to remove contaminations). This is usually done by hydrolyzing siloxane bonds to the more reactive silanol groups at the surface with a strong base. A comparison study of almost one hundred articles concerning monolithic columns in capillaries found that the etching methods (pre-treatment) were diverging, but three procedures were found to be representative for most of the methods [56]. It was shown that a longer treatment at higher temperatures gave a higher percentage silanol groups at the surface than a treatment at room temperature. The increased roughness after etching (Figure 4) also gave a better attachment of the polymer after silanization than the smooth surface.

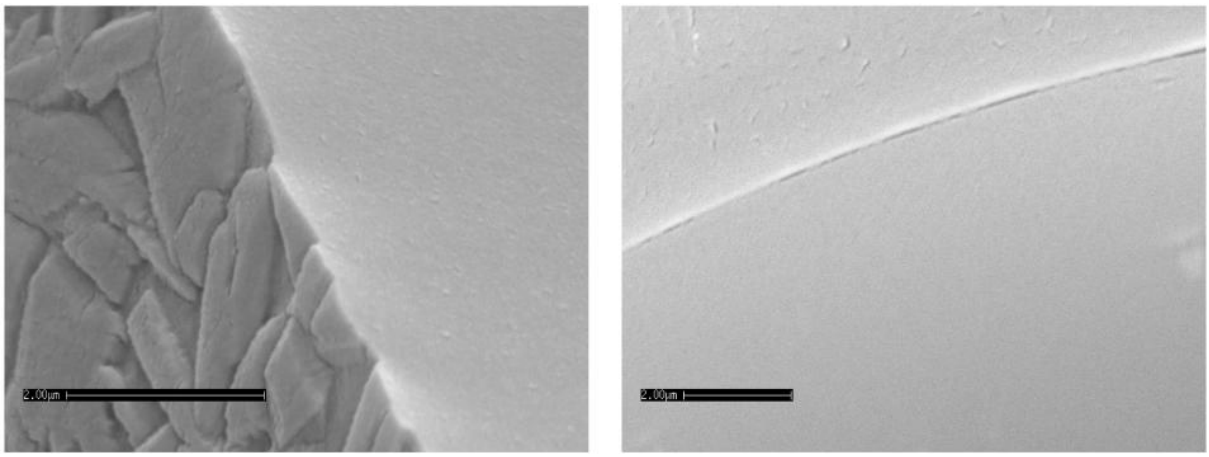

Figure 4. Scanning electron micrographs showing the smooth inner surface of an untreated silica capillary (right) and the inner surface of a capillary treated with 1M NaOH at 120°C for 3 hours (left). The scale bar, representing 2 µm, being placed above the inner surface. Figure reprinted from [56].

4.8.2 Silanization

Silanization is the process where a reactive group is attached to the fused silica surface. The most common method involves the introduction of vinyl groups with the reagent 3-(trimethoxysilyl)propyl methacrylate (γ-MAPS) (Figure 5).

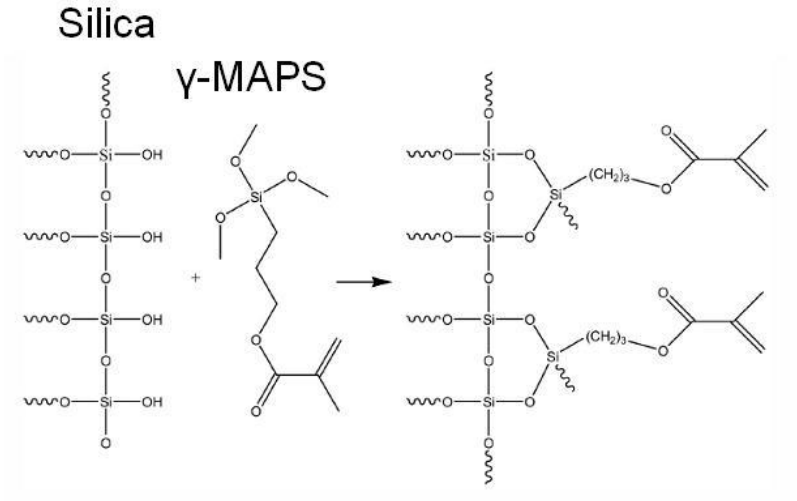

Figure 5. The reaction between fused silica and γ-MAPS. Figure adapted from [56].

This reaction is carried out for two reasons. First, the introduced vinylic groups serve as anchoring sites for the polymer that prevents formation of void channels and excretion of the monolith from the column [57]. Secondly it ensures complete coverage of the inner wall with a polymer layer (Figure 6), this is important to prevent adsorption of analytes during chromatography.

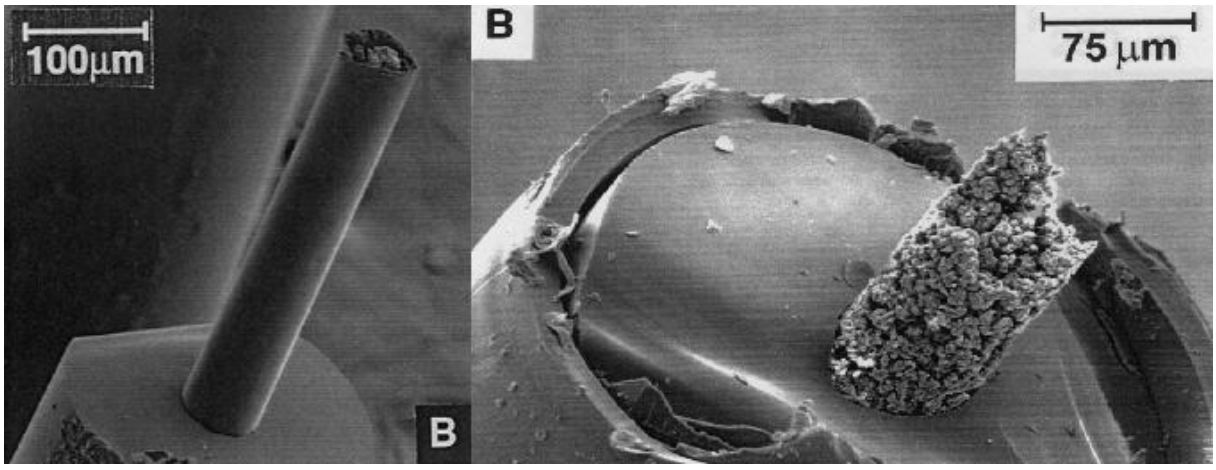

Figure 6. The effect of surface coverage with polymer on the capillary inner wall with silanization (left) and without silanization (right). Figure adapted from [57].

The reaction of the silanizing agent γ-MAPS with the silanol groups at the fused silica surface is preferentially carried out at elevated temperatures. However, at high temperatures auto-polymerization of the reagent via the vinyl groups occurs, resulting in partially filling of the cross section with γ-MAPS (Figure 7, right) or void channels between the monolith and the capillary inner wall after polymerization (Figure 7, left). In order to slow down the polymerization reaction, the inhibitor 1,1-diphenyl-2-picrylhydrazyl (DPPH) is added to the mixture [58].

In the previous mentioned comparison study of Courtois et al. [56] eleven different widely used silanization procedures were examined. It was shown that removing water before the silanization is critical for covalent attachment with the capillary wall, and it was concluded that only a few procedures published in literature gave a good pre-treatment and silanization, and some of the most frequently cited protocols gave non-satisfactory results.

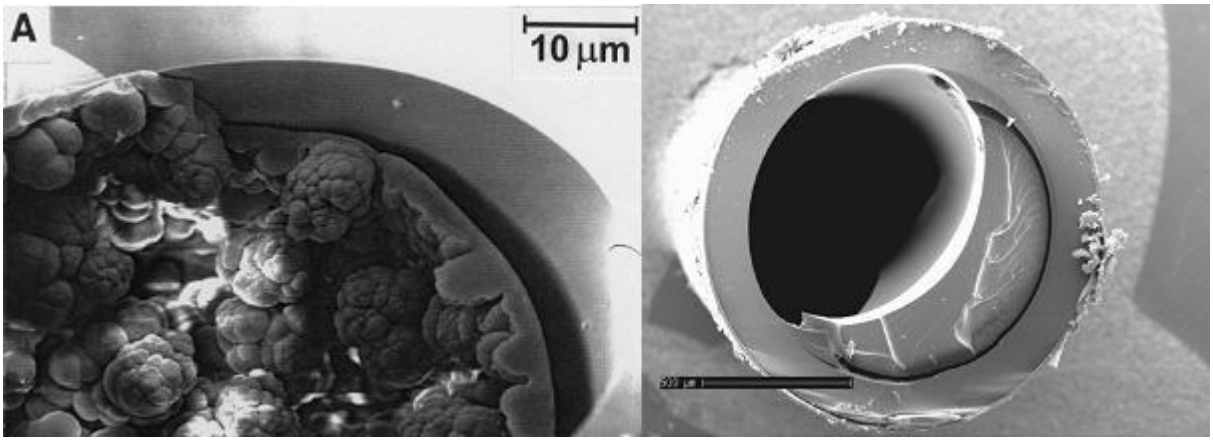

Figure 7. The effect of DPPH on the silanization. The inner wall was silanized without an inhibitor (left) followed by polymerization. The wall was silanized with too high γ-MAPS/DPPH concentration ratio, leading to auto-polymerization of the γ-MAPS (right, the scale bar, representing 500 µm). Reprinted from [57] (left) and [56] (right).

4.8.3 Polymerization

The last step in the preparation of monolithic columns is the polymerization step. The capillary is filled with a solution consisting of monomer (one must be a cross-linker), an initiator and a mixture of solvents. The mixture of solvents consists of a good solvent for the monomers (porogen) and a poor solvent for the monomers. The organic polymer monoliths

can be divided into three groups according to the monomers used; polystyrene, polyacrylamide and acrylate-,methacrylate-based monoliths [59]. The polymerization step is usually initiated by the radical initiator azobisisobutyronitrile (AIBN) which either can be initiated by ultraviolet (UV) radiation or by heat. A heat initiated polymerization is usually carried out at 60-80°C for 16-20 hours [59]. The advantage with UV polymerization is the short polymerization time of about ten minutes. The disadvantages are the need of UV-transparent tubing and a strong UV-source [60].

The choice of porogen is the most common tool to control the porous properties without changing the chemical composition of the final monolith [53]. Viklund et al. have shown how the pore size distribution is affected by temperature, porogenic solvent and amount of cross-linker [54]. An increase in polymerization temperature resulted in larger pores because a higher temperature gives a higher nucleation rate. When more nucleuses and globules are formed and the amount of monomer is the same, the result is more and smaller globules. The polymer phase separates from the solution during polymerization because of its limited solubility in the polymerization mixture that results either of both from a molecular mass that exceeds the solubility limit or from insolubility derived from cross-linking. Adding a poorer solvent for the monomers gave larger pores, since polymers precipitate earlier from the solution, and the polymerization reaction proceeds mainly in the larger polymer globules. The globules formed in such a system are larger and, consequently, the voids between them (pores) are larger as well. An increased amount of cross-linking monomer led to earlier precipitation, but true coalescence did not occur. Therefore the globules (and pores) became smaller. The amount of cross-linker also affects the final monolith (e.g. degree of swelling).

Polymerization is also affected by the surface coverage of γ-MAPS. If the surface treatment has been successful in introducing a high density of methacrylic groups, the concentration of growing polymers on the surface may exceed the bulk solution. Then a dense layer of polymer is made on the surface and less monomer is available to form the monolith (Figure 8). This effect is larger in smaller i.d. capillaries, and requires time monitoring when a continuous flow is used in the silanization step [61]. There might also be a depletion of monomer close to the wall, leading to a lower mechanical strength of the monolith and excretion from the column, even with proper silanization [56].

The major disadvantage with monoliths is the great care required in the preparation of these columns, especially when smaller i.d. columns are prepared, due to the larger surface area to i.d. ratio.

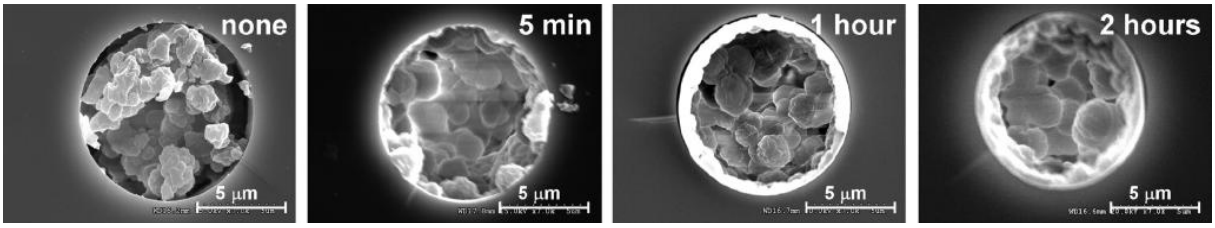

Figure 8. SEM images of the cross section of monoliths in 10 µm i.d. fused silica capillaries modified with γ-MAPS for different periods of times, followed by polymerization. Reprinted from [61].

4.9 Porous layer open tubular (PLOT) columns

There have been several attempts to use open tubular columns in liquid chromatography, but the success has been limited [62,63]. A breakthrough for PLOT columns in LC came in 2007, when Karger and his group successfully prepared and used a 10 µm i.d. PS-DVB column [27]. The fabrication of this column followed the same procedures used for monoliths except the monomer solvent was changed from a porogenic mixture to a single solvent, giving an polymer precipitation at an early stage in the polymerization process [54,64], forming a thin porous layer at the capillary wall, while leaving open the main section of the capillary tube [27]. The high permeability of the PLOT column allowed the use of a 4.2 m long column with the use of conventional HPLC pumps. The columns provided a high efficiency separation of small and larger peptides, and both the run-to-run and column-to-column repeatability were high. The same group later included this PLOT column in a more advanced setup used on peptides, and also made a PLOT hydrophilic interaction ion chromatography (HILIC) column for carbohydrate separation [65,66].

4.10 Aim of study

The purpose of this study was to prepare PLOT poly(styrene-divinylbenzene) columns and use these columns to separate intact proteins with gradient elution and MS detection. The effect of temperature and the column-to-column and run-to-run retention time repeatability were examined. One of the goals was also to assess the potential of coupling the PLOT column to an immobilized on-line trypsin reactor.

5. EXPERIMENTAL

5.1 Materials and reagents

Divinylbenzene (DVB) 80% mixture of isomers, styrene (99%), the initator AIBN and inhibitor DPPH were purchased from Sigma Aldrich (St.Louis, MO). Carbonic anhydrase, myoglobin, cytochrome C, β-lactoglobulin A, β-lactoglobulin B, bovine serum albumin and ovalbumin, substance P, methionine-enkephalin (Met-enkephalin), leucine-enkephalin (Leu-enkephalin) were purchased from Sigma Aldrich. Urea (>99.5%), trifluoroacetic acid (TFA) (99%), formic acid (FA) (50%) were purchased from Fluka (Sigma Aldrich). "Ultrapure" tris(hydroxymethyl)aminomethane (tris) was purchased from Gibco BRL/Invitrogen (Carlsbad, CA). DTT (DL-dithiothreitiol), ammonium acetate, triethylammonium bicarbonate buffer 1M (pH 8.5), γ-MAPS (98%), N,N-dimethylformamide anhydrous (DMF) and iodacetamide (IAM) were purchased from Sigma Aldrich. Polyimide coated fused silica tubing (360µm outer diameter (o.d.), 10, 50 and 5 µm i.d.) was purchased from Polymicro Technologies (Phoenix, AZ). Tetrahydrofuran (THF) (>99.7%), butylacetate (99.5%) and 1-propanol (>99.7%) were purchased from Merck (Darmstadt, Germany). Toluene (glass distilled grade) was obtained from Rathburn (Walkerburn, UK). Ethanol and anhydrous ethanol were purchased from Arcus (Oslo, Norway). Nitrogen (99.99%) was obtained from AGA (Oslo, Norway). Grade 1 water was produced with a Milli-Q Ultrapure water system (Millipore, Bedford, MA).

5.2 Column preparation

The columns were prepared as described by Yue et al. [27] with only a few practical deviations. The solutions were weighed using HPLC syringes. A home made pressure bomb connected to a nitrogen flask was used to fill and wash the capillaries in all steps (Figure 9). Nitrogen gas was filled into the pressure bomb and forced the liquid through the capillary; hence the pressure used in the different steps was equal to the gas pressure, and ranged from 110 to 200 bar. Fused silica tubing (10 µm i.d.) was filled with 1M NaOH and the ends were plugged (with ferrules, nuts and steel caps) and left overnight. The capillaries were

subsequently washed with water and acetonitrile followed by drying with nitrogen to remove water and residual acetonitrile. A solution consisting of thirty percent (v/v) γ-MAPS and (0.5% (wt/v) DPPH in DMF was freshly prepared and filled into the 10 µm i.d. pre-treated capillary. Both ends were plugged, and the capillary was placed in an oven at 110°C for 6 hours. The capillary was flushed with acetonitrile and blown dry with nitrogen. A polymerization solution containing 5 mg of AIBN, 200 µL of styrene, 200 µL of DVB, and 600 µL of ethanol (96%) was prepared (if not otherwise stated). The solution was degassed by ultrasonification for 5 minutes and then filled into the silanized capillary. Both ends were plugged, and the capillary was heated at 75°C for ~ 16 hours. The column was washed with acetonitrile and ready for use. An additional drying step with nitrogen was used if SEM images were to be taken, or viewed in a microscope. During this drying step nitrogen was blown through the column and into acetonitrile to ensure that the column was open.

Additionally a 5 µm i.d. capillary was used to produce a PLOT column following the same procedure as for the 10 µm i.d. capillaries. Other solvents were also used for preparation of 10 µm i.d. PLOT columns, and these were methanol, butylacetate, toluene and anhydrous ethanol. A monolithic 50 µm i.d. was also prepared, following the same procedures as for the PLOT columns except for the polymerization mixture. A polymerization solution containing 5 mg of AIBN, 200 µL of styrene, 200 µL of divinylbenzene, 530 µL of 1-decanol and 70 µL of THF [20] was made and filled into the 50 µm i.d. silanized capillary.

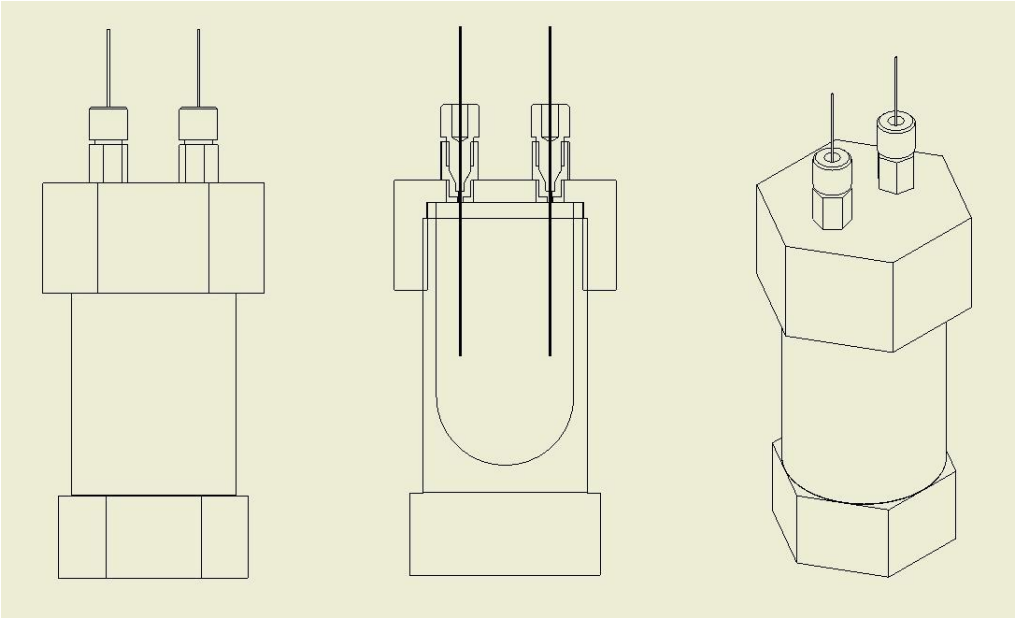

Figure 9. Schematic drawing of the home-made pressure bomb used in the preparation of the PLOT columns. Figure by Inge Mikalsen.

5.3 Sample and standard preparation

5.3.1 Preparation and storage of protein standards

Protein standards were dissolved in water to a concentration of 0.1 mg/mL and stored in aliquots at - 18°C. Defrosted solutions were not used for more than 2 weeks. Protein mixtures were freshly prepared each morning. Peptides were dissolved in water and stored at + 4°C.

5.3.2 Reduction and alkylation of β-lactoglobulin A and B

For reduction and alkylation a previously reported procedure was used [67]. Briefly, 1 mg of protein was dissolved in 1 mL of 4 M urea and 0.1 M tris (pH 8.5). 10 µL of 1 M DTT was added to the protein solution which was incubated for 120 min at 37 °C followed by cooling to room temperature before adding 20 µL of 1M IAM for alkylation, whith continued incubation for 70 min in the dark. Subsequently, 40 µL of 1M DTT was added for quenching the alkylation.

5.3.3 Trypsination of cytochrome C

For trypsination, 100 µL of 1.0 mg/mL cytochrome C was dissolved in 0.5 M triethylammonium bicarbonate buffer (pH 8.5) and 10 µL 1 mg/mL TPCK treated trypsin (aq) (bovine pancreas, Sigma) was added. The solution was treated in a homemade infrared radiation (IR) oven similar to that described elsewhere for 5 minutes at 37°C [68].

5.3.4 Preparation of milk sample

2 µL formic acid was added to 200 µL skimmed milk (0.1% fat). 100 µL of water was added and the mixture was centrifuged for 5 minutes at 12 000 revolutions per minute. The supernatant was used for analysis.

5.4 SEM procedure

SEM pictures were obtained using a FEI Quanta 200 FEG-ESEM (FEI, Hillsboro, OR). The capillaries were cut into pieces of approximately 1 cm and placed on a standard carbon tape. The images were taken at low vacuum and a large field detector (LFD) was used. The other parameters differed and are stated under each image.

5.5 The LC-MS system

The LC-MS system used is shown in Figure 10. An 1100 series Agilent pump with a G1379A series degasser (Agilent, Sao Paulo, CA) was set to 2 µL/min. Samples were injected using a VICI injector (Valco Instruments, Houston, TX) with an internal loop volume of 500 nL. The eluent was split just after the injector with a ratio of 1/100 using a Valco T-piece, so that 20 nL/min entered the PLOT column (same flow rate as used by Yue et al. [27]), and the main part to waste. Hence, the effective injection volume was 5 nL. Flow rates of the PLOT column were measured by connecting 50 µm i.d. open fused silica capillary to the exit of the PLOT column, and measuring the volume of the mobile phase that

flowed for a given period of time. A chosen flow rate was obtained by varying the length of the 15 µm i.d. fused silica capillary from the split (T-piece) to waste. After obtaining a measured flow rate between 19.7 and 20.3 nL/min the PLOT column was connected to a PicoTip™ nanospray tip (360 µm o.d., 20 µm i.d. with 5 µm i.d. spray tip) with a Picoclear™ union, both purchased from New Objective (Woburn, MA). For MS detection, an LCT time of flight (TOF)-MS (Waters, Milford, MA) was employed and operated in positive ionisation mode; the capillary, sample cone and extraction cone voltages were set to 1200, 30 and 10 V, respectively. The RF lens was set to 500 units and the source temperature was set to 120°C. Scan rate was 1 scan/sec, and the mass to charge ratio (*m/z*) range was 650-1650 except for analysis of milk where the upper limit was extended to 2100. For temperature control a Mistral LC-oven (Spark-Holland, Emmen, Netherlands) was used. The MS was controlled by MassLynx version 4.1 (Waters).

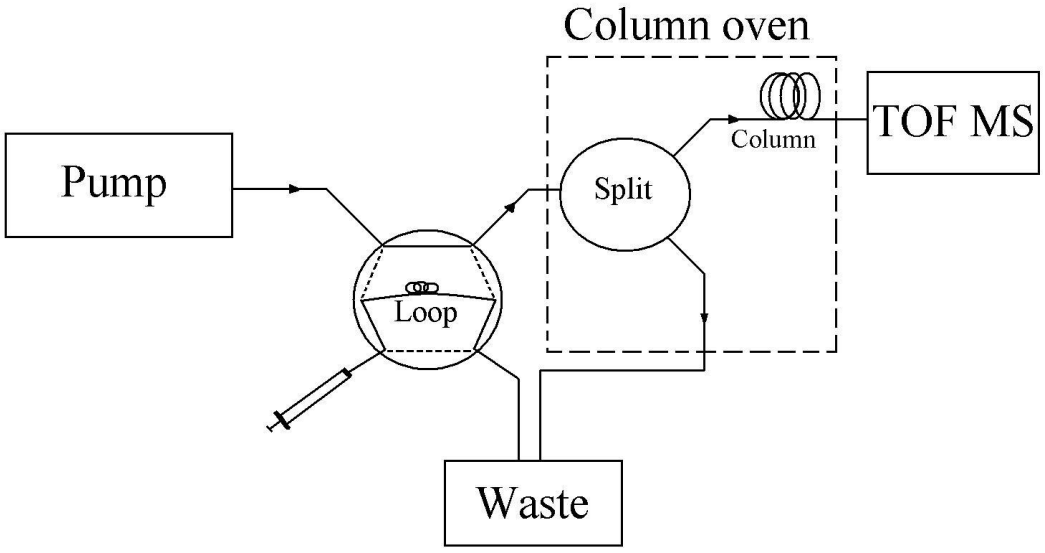

Figure 10. The LC-MS system.

6. RESULTS AND DISCUSSION

6.1 Preparation of PLOT columns

The columns were prepared with ethanol as solvent for the monomers as described in the experimental section. Yue et al. reported that large globules were seen in the end sections of their 5 m long prepared column, and therefore 40 cm was cut from both sides [27]. SEM images (Figure 11) and optical microscopy (2000x) of the prepared PLOT columns, showed that large globules were formed not only at the ends, but were evenly distributed throughout the 3 m long columns made in the present study.

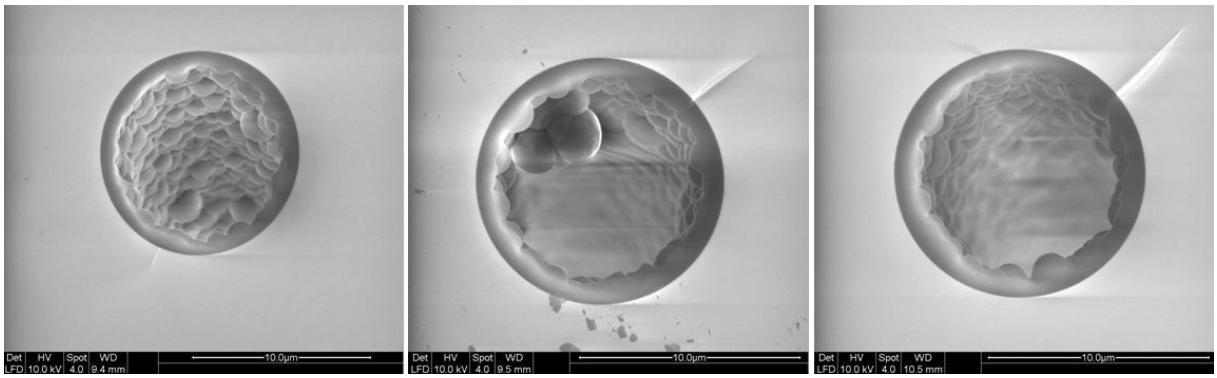

Figure 11. SEM images of a PLOT column prepared with ethanol as solvent for the monomers. The images are taken 10 cm from both ends (left, right) and in the middle (middle) of a ~3 m long column.

In a later study by Karger's group, in the preparation of PLOT column with vinylbenzyl chloride (instead of styrene), methanol was chosen as the monomer solvent [66]. To examine if any column improvement could be made (less/smaller globules and a more even layer), a column was prepared with methanol instead of ethanol as the monomer solvent (Figure 12). The globules formed were found to be larger with methanol than ethanol as solvent. This is in agreement with the effect observed for monoliths where larger globules are formed when poorer monomer solvents are used [54].

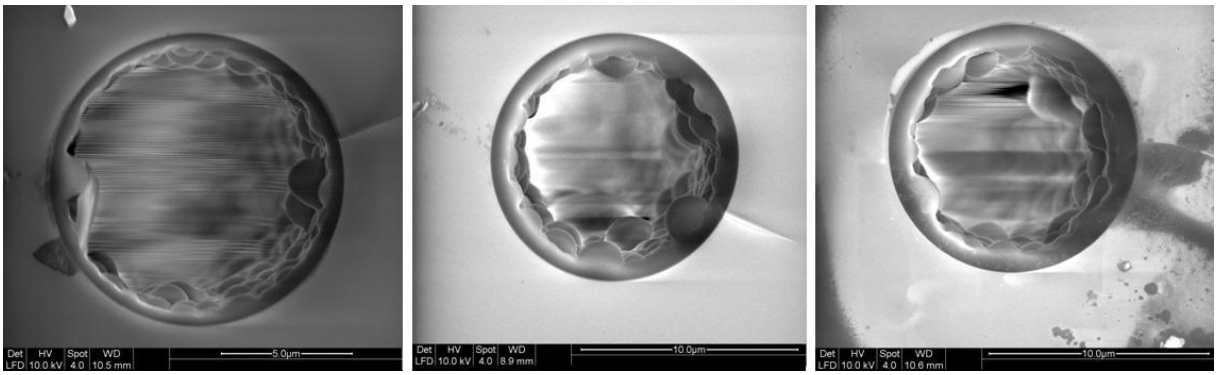

Figure 12. SEM images of a PLOT column prepared with methanol as solvent for the monomers. The images are taken from 10 cm from both ends (left, right) and in the middle (middle) of a ~3 m long column.

Visual inspection of several columns by microscopy showed that from 5 to 15 cm of the ends were either not filled, or partially filled with polymer (Figure 12, left). It was concluded that the ends should be cut off to ensure an even layer of polymer throughout the whole column. For further column preparation investigation shorter capillaries of 1 m was used due to ease of handling.

6.1.1 Polymerization temperatures

To evaluate the robustness of the column polymerization method and hopefully decrease the amount of globules formed, polymerization at different temperatures were carried out. Table 1 shows open capillaries can be made over a temperature range of 20°C. For some monolithic columns this range can be smaller than 10°C [69]. No visual differences could be observed by microscopy of the PLOT columns polymerized at different temperatures, and therefore the original temperature of 75°C was kept.

Table 1. Temperature effect on polymerization. Clogged implies cross polymerization in the capillary while open implies wall polymerization only. Clogged or open columns were verified by nitrogen pressurization.

Temperature	Time	Clogged/Open
90	16	Clogged
85	16	Open
75	16	Open
65	24	Open

6.1.2 Monomer solvents

Other monomer solvents than that used by Yue et al. [27] were also examined in order to investigate polymerization solution robustness and hopefully decrease the globule size. The different solvents used are shown in Table 2. Yue et al. reported that more than 50% monomer in the polymerization mixture resulted in clogging of the capillary [27]. This is in agreement with 12% (v/v) of toluene in the polymerization mixture (12% toluene + 40% monomer = 52%) since toluene resembles the monomers used, and can be compared to 50% monomer in the polymerization mixture leading to clogging.

Table 2. Effect of solvent on column preparation. The percentage of each solvent is given as (v/v) in ethanol. See Table 1 for definition of clogged/open.

Solvent	Clogged/Open
6% Toluene	Open
6% THF	Open
6% Butylacetate	Open
12% Toluene	Clogged
12% THF	Partially clogged
12% Butylacetate	Open
Anhydrous ethanol	Open
1-Propanol	Clogged

No improvement was seen with anhydrous ethanol compared to ethanol as monomer solvent (Figure 13, middle). Addition of Butylacetate in the polymerization mixture gave a more uneven poly(styrene-divinylbenzene) layer with more globules. Using other hydrophobic solvents makes the columns more prone to clogging, the preparation of columns using a single solvent as monomer solvent is easier and more repeatable than using a mixture of

solvents and since the columns prepared with ethanol worked well in chromatography (shown later), ethanol was kept as the monomer solvent.

Other columns prepared but not used chromatographically were a 5 µm i.d. PLOT column prepared using ethanol as solvent and a 50 µm i.d. monolithic column using the same steps as for the PLOT columns, only changing monomer solvent to a mixture of 1-decanol and THF (Figure 13, left, right). Both columns were pressurized with nitrogen and gave flow through.

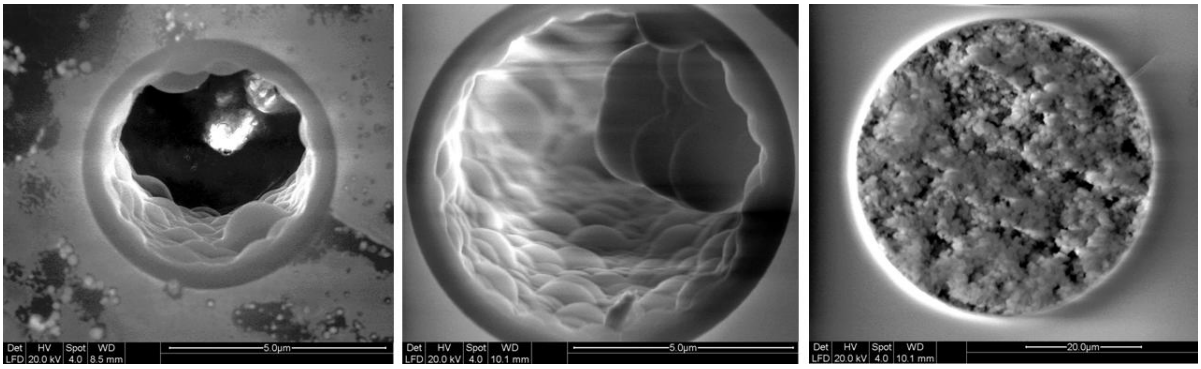

Figure 13. SEM images of 3 different columns. PLOT columns polymerized in a 5 µm and 10 µm capillary with ethanol and anhydrous ethanol as solvent (left and middle respectively). A monolithic column prepared in a 50 µm capillary using the same conditions as the PLOT columns, only changing monomer solvent (right).

6.1.3 Polymer attachment

During this work voids between the polymer and the capillary inner wall were never observed. Therefore the silanization procedure used was found to be adequate. However, if problems with the mechanical stability of PLOT columns at higher pressures occur, a new silanization procedure should be examined. Even though the silanization in DMF has shown to be satisfactory (50% γ-MAPS, not 30%), the silanization in toluene (10% γ-MAPS) gave a much higher surface coverage, and higher mechanical stability after polymerization [56]. The effects of high surface coverage of γ-MAPS in monolithic columns discussed earlier are not a problem for PLOT columns; only for small i.d. monolithic columns (Figure 8). The high γ-

MAPS surface coverage might be an advantage due to less chance of clogging during polymerization.

6.2 Selection of protein standards and limitations

After some initial experiments with peptide standards, separation of protein standards was carried out. The proteins used for PLOT column experiments are shown in Table 3. However due to limitations with the detection of proteins of high molecular masses (Figure 14), the upper mass limit for protein detection was found to be the 29 kDa carbonic anhydrase.

Table 3. The molecular mass of the protein standards used.

Protein		Mass (kDa)
Cytochrome C	Cyt C	12
Myoglobin	Myo	17
β-lactoglobulin A	β –A	18
β-lactoglobulin B	β –B	18
Carbonic anhydrase	CA	29
Ovalbumin	Ova	44
Bovine serum albumin	BSA	67

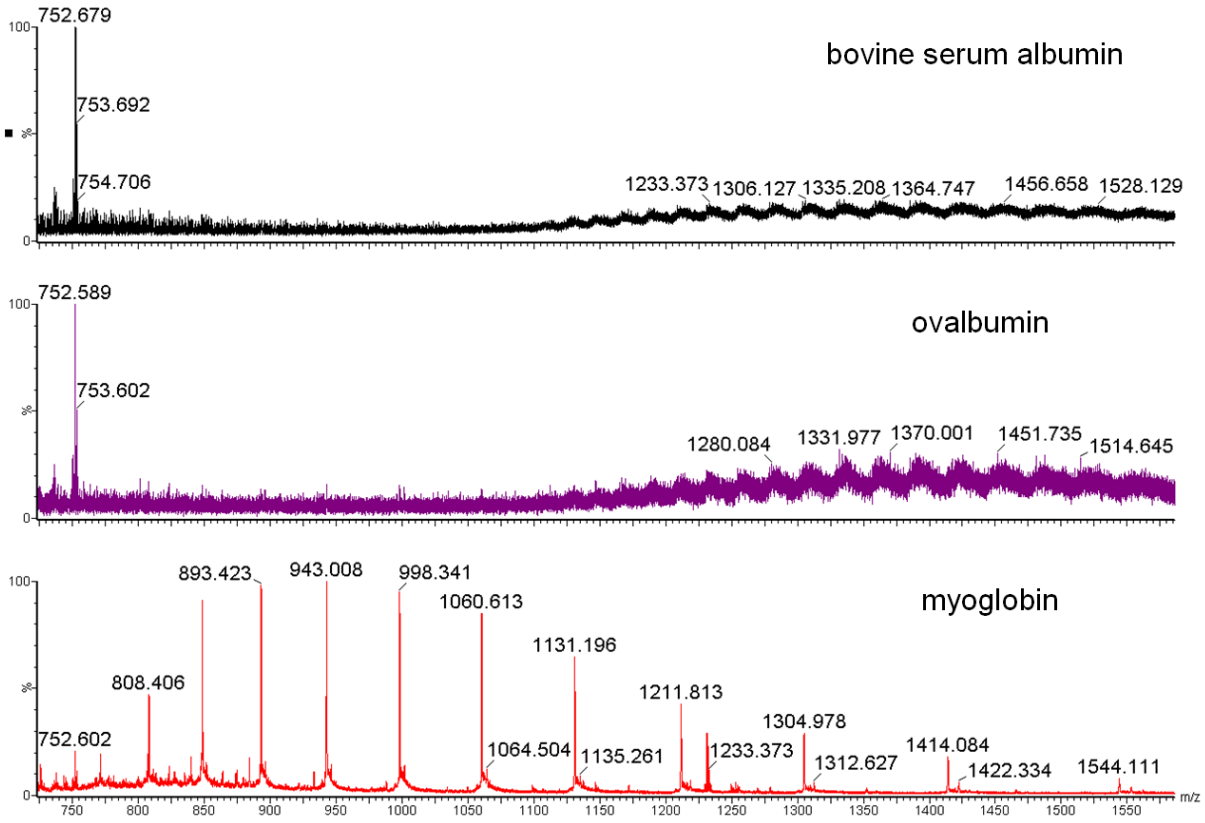

Figure 14. Mass spectrum of bovine serum albumin (top), ovalbumin (middle) and myoglobin (bottom) from direct injection of 50 µg/mL protein in 0.1% FA (v/v) in 50% water in acetonitrile (v/v). Each mass spectrum is scaled relative to the highest intensity signal.

6.3 Mobile phase additives

Trifluoroacetic acid is one of the most popular reagents added for peptide and protein separation, because of improved peak shape and smaller peak widths [70]. In LC-MS it is less commonly used because of ion suppression [71]. However addition of 0.05% to 0.1% (v/v) of TFA has been used to improve peak shape and reduce peak widths in LC-MS analysis [72,73]. Since signal suppression is dependent of flow and instrumentation used, the signal suppression, peak width and peak shape were examined with and without addition of TFA (Figure 15). Since the loss of intensity was less than 50% and the width of the peaks were significantly improved 0.05% TFA was added to the mobile phase (in addition to 0.1% FA) in all later experiments.

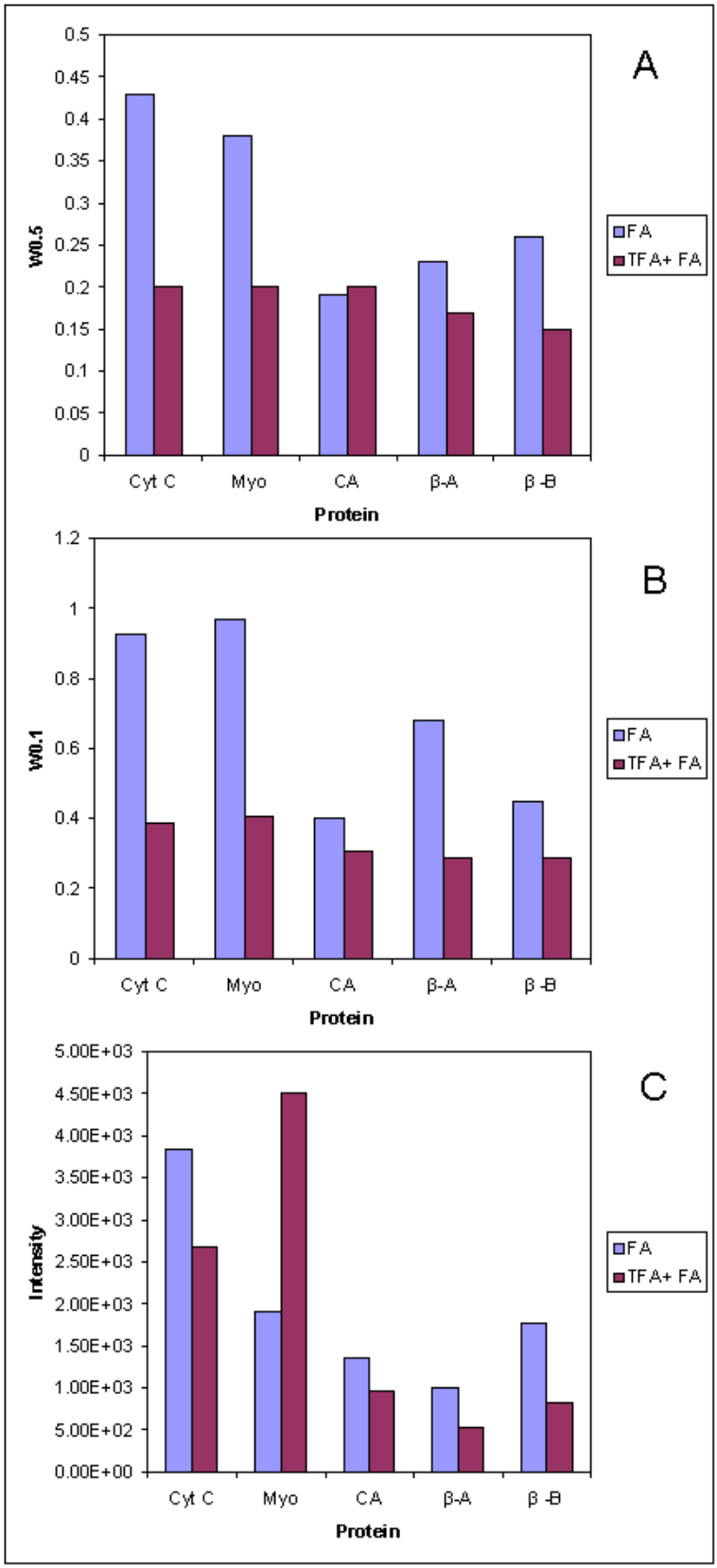

Figure 15. Peak width and intensity using a solvent gradient from 100% A (water and mobile phase additive) to 90% B (10% water in acetonitrile and mobile phase additive) where mobile phase additive were either 0.1% FA or 0.05% TFA and 0.01% FA. Injected concentration was 10 µg/mL of each protein.

6.4 Column performance

6.4.1 Peak shape vs. concentration of protein

To see how the peak shape was affected by protein concentration, cytochrome C was chosen as model protein since it had the lowest limit of detection (LOD). A low LOD enables analysis of the proteins at sufficiently low concentrations before the loading capacity is exceeded. Injected concentrations were from 1 µg/mL to 100 µg/mL corresponding to 5 pg to 500 pg protein loaded on column. The peak shape was found to be relatively little affected by the concentration cytochrome C within the concentration range injected (Figure 16 and Figure 17). However the peak becomes broader at concentrations above 20 µg/mL, corresponding to 100 pg protein.

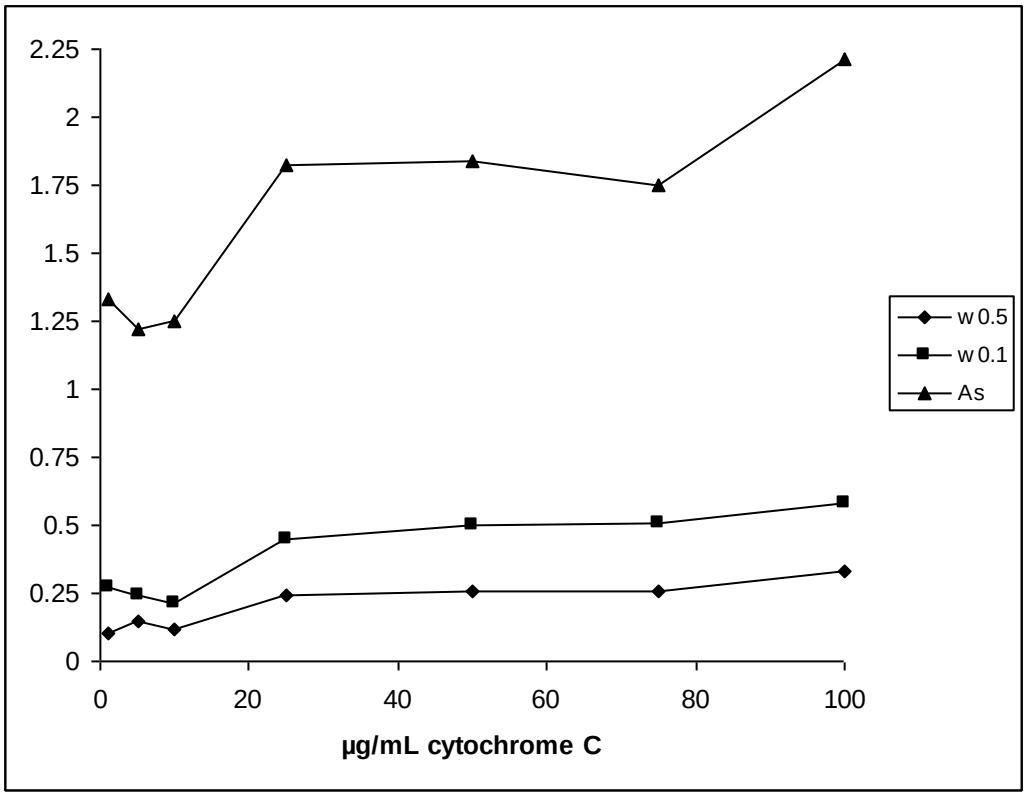

Figure 16. Peak width at 10% and at 50% of the peak height and the peak asymmetry ($w_{0.1}$, $w_{0.5,}$ As respectively) vs. concentration of the injected sample. The solvent gradient was 90 % A (0.1% FA, 0.05% TFA (v/v) in water) to 90 % B (0.1% FA, 0.05% TFA, 10% water (v/v) in acetonitrile) in 40 minutes.

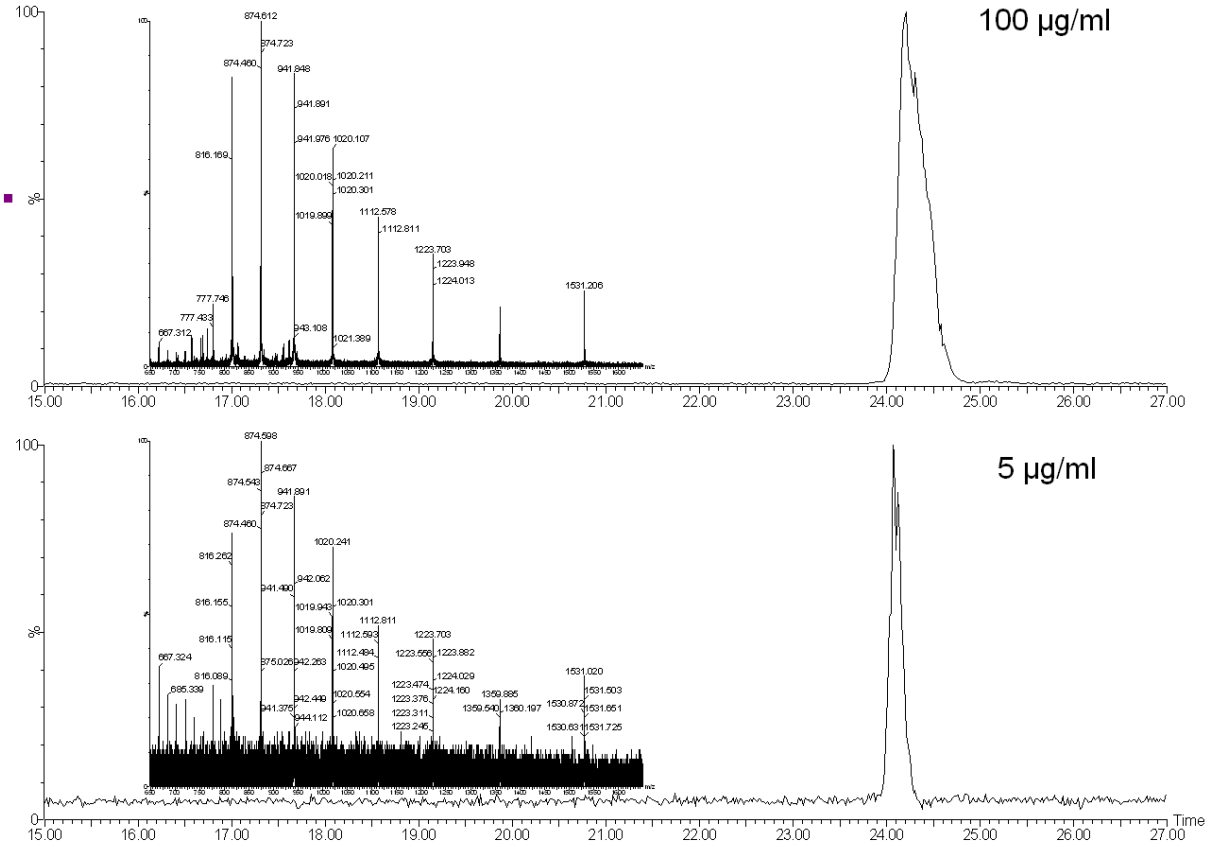

Figure 17. Extracted ion chromatogram (EIC) of 5 µg/mL and 0.1mg/mL cytochrome C with mass spectrum included. Conditions were as in Figure 16. Each chromatogram and mass spectrum is scaled relative to the highest intensity signal, which was set to 100.

6.4.2 Run-to-run retention time repeatability

To assess the run-to-run retention time repeatability, a solution containing three proteins each at a concentration of 33µg/mL each was injected five times. At first a 15 minute conditioning step between each gradient was used (~2 column volumes). This gave a shift in retention time between each analysis (results not shown). A 30 min conditioning step at 30 nL/min between each gradient gave stable retention times (Figure 18). The relative standard deviation (RSD) of the retention time of the standard proteins was below 0.6 % (Table 4) which was half the value obtained by Yue et al. [27].

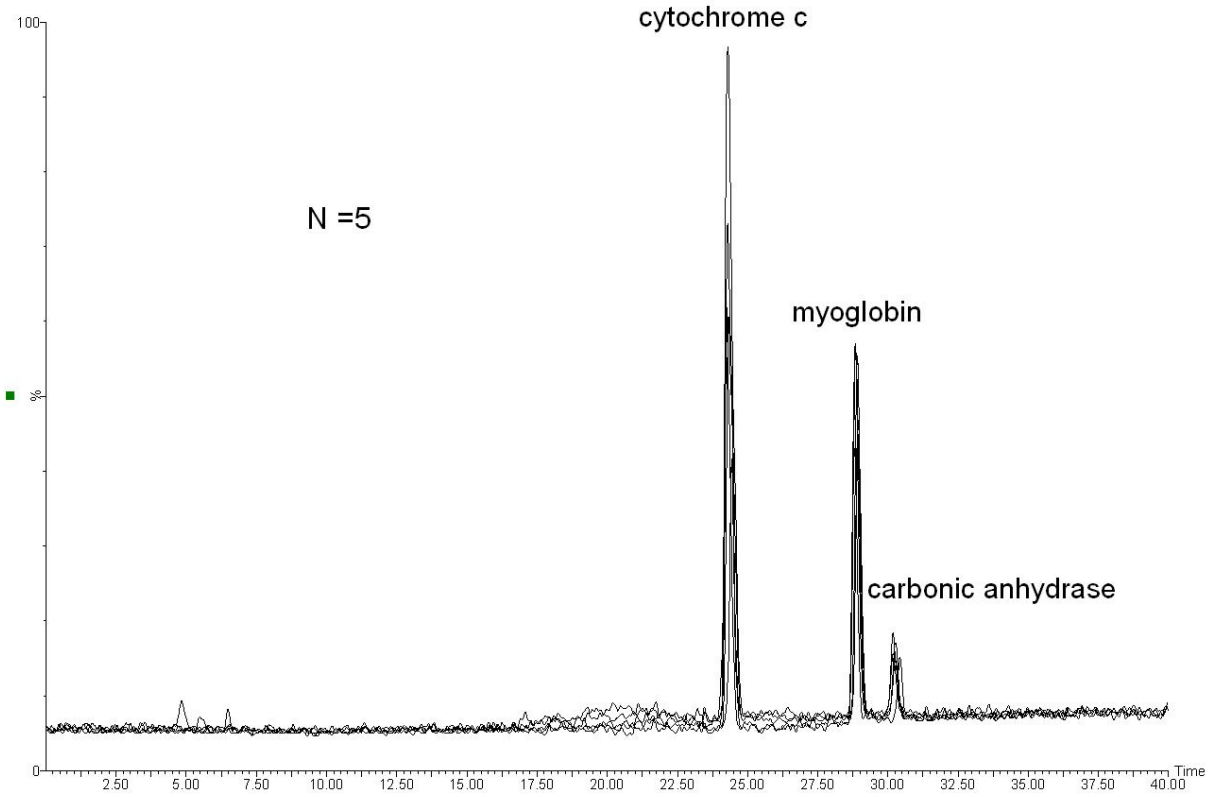

Figure 18. Overlay base peak intensity (BPI) chromatograms of five consecutive separations of three proteins. Injected concentration was 33 µg/mL of each protein. Conditions were as in Figure 16.

Table 4. Retention times for 3 proteins from 5 consecutive separations.

Run	Cyt C	Myo	CA
1	24.30	28.84	30.19
2	24.30	28.85	30.19
3	24.32	28.91	30.27
4	24.43	28.93	30.27
5	24.25	28.82	30.43
AVG	**24.28**	**28.83**	**30.31**
STD	**0.04**	**0.01**	**0.17**
RSD	**0.15**	**0.05**	**0.56**

6.4.3 Column-to-column repeatability

Three columns that were made separately showed good repeatability regarding t_R, peak width at 50% and 10% of peak height ($w_{0.5}$ and $w_{0.1}$ respectively) and asymmetry (As) of the three standard proteins (Figure 19 and Table 5). The $w_{0.5}$ was typically 0.2 minutes. The operating pressures were also similar. Column one and two gave a back pressure of 192 bar and column three of 185 at a flow of ~20 nL/min (10% B). The column-to-column repeatability

with respect to retention times was 2.5% and lower. This is in agreement with Yue et al. who reported an average retention time RSD (13 peptides) of 2.5% [27]. In comparison column-to-column retention time repeatability on commercially available columns can be as high as 3.7%. The relative standard deviation between column assemblies was not examined. Hence the variations in retention times can also be explained by differences in flow measurements, emitter attachment and partial clogging of the emitter.

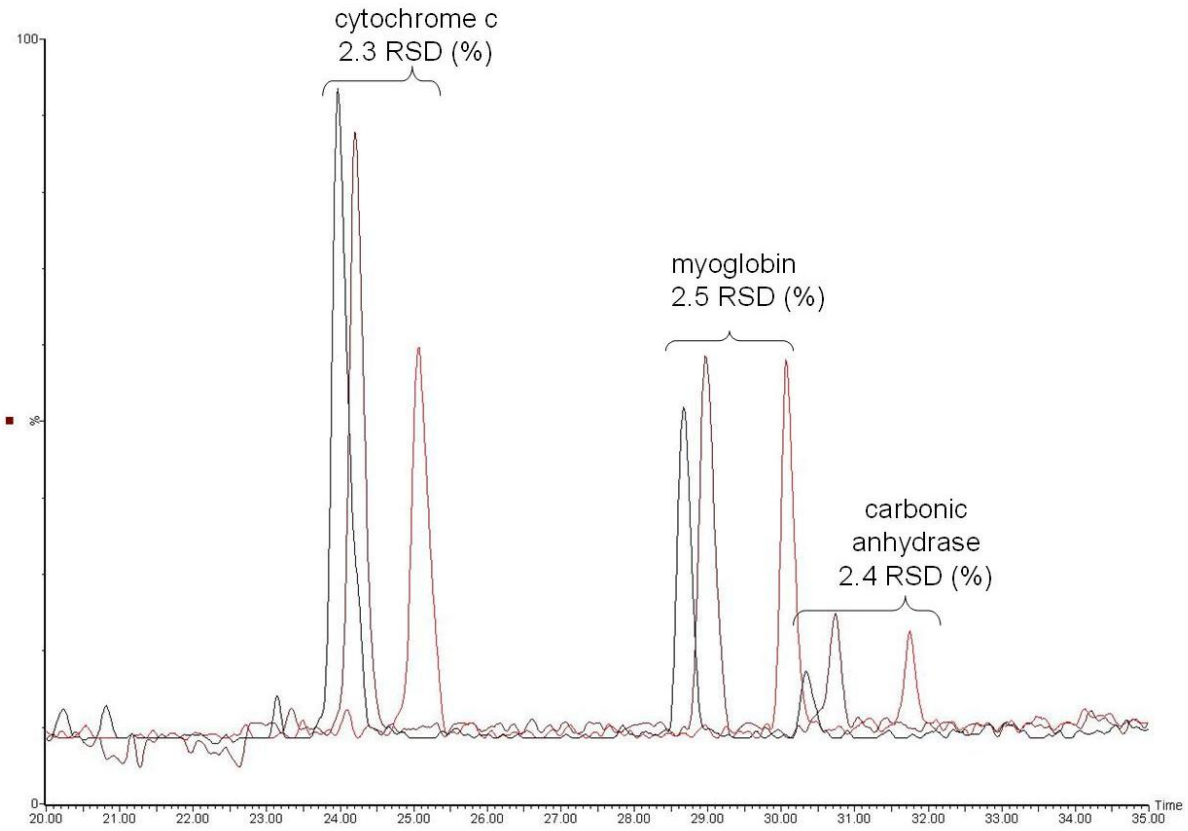

Figure 19. Overlay BPI chromatograms for 3 separately prepared columns. Standard deviation of the retention times are shown above the peaks of each protein. Injected concentration was 33 µg/mL of each protein. Conditions were as in Figure 16.

Table 5. Between-column performance (n =3) of retention time, peaks widths and peak asymmetry.

	t_R			$W_{0.5}$			$W_{0.1}$			As		
Protein	**Cyt C**	**Myo**	**CA**	**Cyt C**	**Myo**	**CA**	**Cyt C**	**Myo**	**CA**	**Cyt C**	**Myo**	**CA**
Col 1	24.19	28.96	30.72	0.2	0.2	0.18	0.42	0.4	0.35	1.41	1.69	1.24
Col 2	25.04	30.05	31.75	0.22	0.19	0.19	0.49	0.34	0.32	1.48	1.29	1.18
Col 3	23.96	28.65	30.32	0.2	0.19	0.2	0.4	0.32	0.34	1.81	1.25	1.44
AVG	**24.40**	**29.22**	**30.93**	**0.21**	**0.19**	**0.19**	**0.44**	**0.35**	**0.34**	**1.57**	**1.41**	**1.29**
STD	**0.57**	**0.74**	**0.74**	**0.01**	**0.01**	**0.01**	**0.05**	**0.04**	**0.02**	**0.21**	**0.24**	**0.14**
RSD	**2.33**	**2.52**	**2.39**	**5.59**	**2.99**	**5.26**	**10.82**	**11.78**	**4.54**	**13.64**	**17.26**	**10.58**

6.4.4 Carry-over

Carry-over was investigated by injecting 0.5 ng of each of protein standard, followed by a blank injection. Carry-over was 0.77%, 1.1% and 0.00% for cytochrome C, myoglobin and carbonic anhydrase respectively. This is an improvement in carry-over for cytochrome C compared with that found using particle packed columns (Appendix I). The largest carry-over observed is shown in Figure 20. It is likely that the carry-over may increase for larger proteins (Appendix I); however, because of the size limitation as addressed earlier, larger proteins could not be examined with the nanospray-MS instrumentation available.

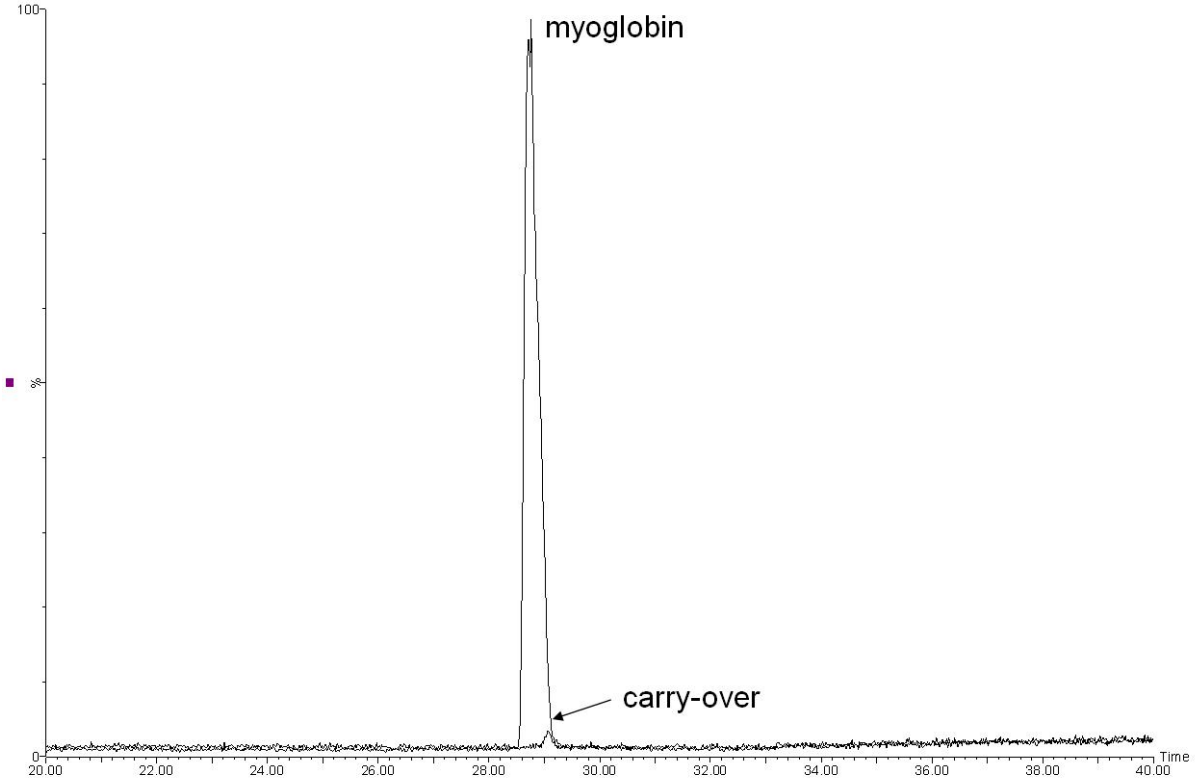

Figure 20. Overlay extracted ion chromatograms (from 20 min) of 0.5 ng injected myoglobin (0.1 mg/mL), and the subsequent blank injection. Conditions were as in Figure 16.

6.5 Effect of gradient time and column length

To assess the effect of gradient time (t_G) on peak width, cytochrome C, myoglobin and carbonic anhydrase were chromatographed from 10 to 90 % B in 20, 40, 60, 90 and 120 minutes. Figure 21 shows a plot of the average relative $w_{0.5}$ as function of t_G for a 3 meter column. The curve is roughly linear, and the smallest $w_{0.5}$ was obtained at t_G 20 minutes (set to value 1) and the largest relative $w_{0.5}$ increase being 2.4 (t_G 120 minutes). In comparison, Wang et al. reported a relative $w_{0.5}$ increase of ~5 for t_G =120 minutes for peptides on a C18 packed column (length 50 mm) [74]. Shorter PLOT columns produced wider peaks, likely due to poorer refocusing or lower capacity.

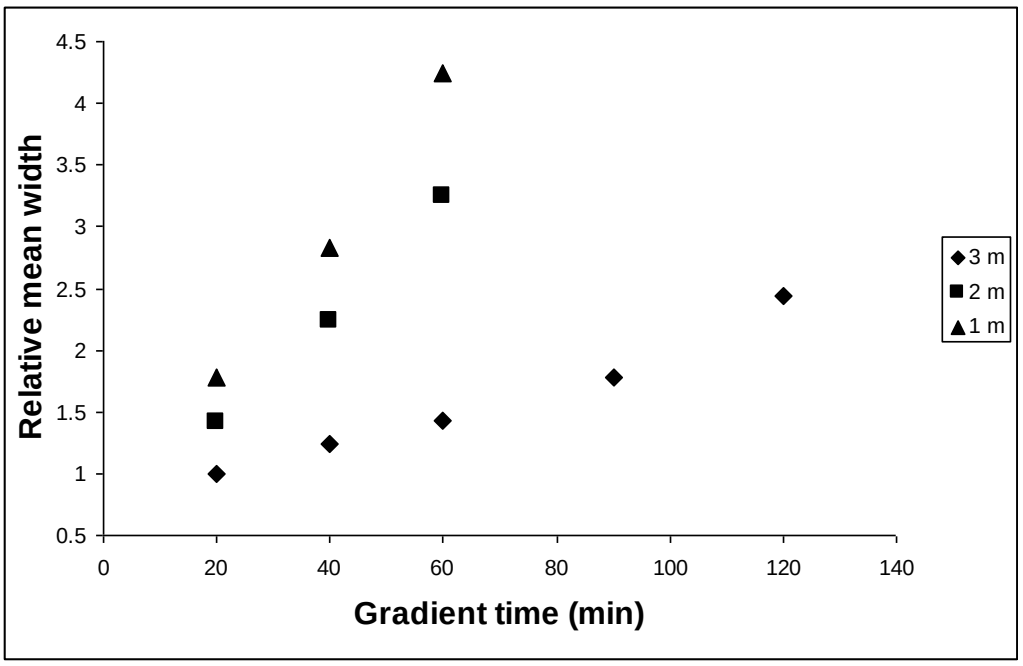

Figure 21. Plot of the average relative $w_{0.5}$ as function of t_G for 1, 2 and 3 meter columns. Smallest average $w_{0.5}$ was 0.14, and set to unity. Injected concentration was 33 µg/mL of each protein. Other conditions were as in Figure 16.

6.6 Effect of temperature

Column efficiency has been shown to increase with temperature [75], but elevated temperatures can potentially alter protein structure and hence their retention. A mixture of the five first proteins shown in Table 3 was chromatographed at 20, 30, 40, 50 and 60° C to examine the effect of temperature. The three closely eluting β-lactoglobulin A, β-lactoglobulin B and carbonic anhydrase were not baseline separated at 20° C (Figure 22, top and Figure 23, top) and the column back pressure was 143 bar. At 40° C, the compounds were fully separated (Figure 22, bottom and Figure 23, bottom), and the column back pressure was reduced to 113 bar.

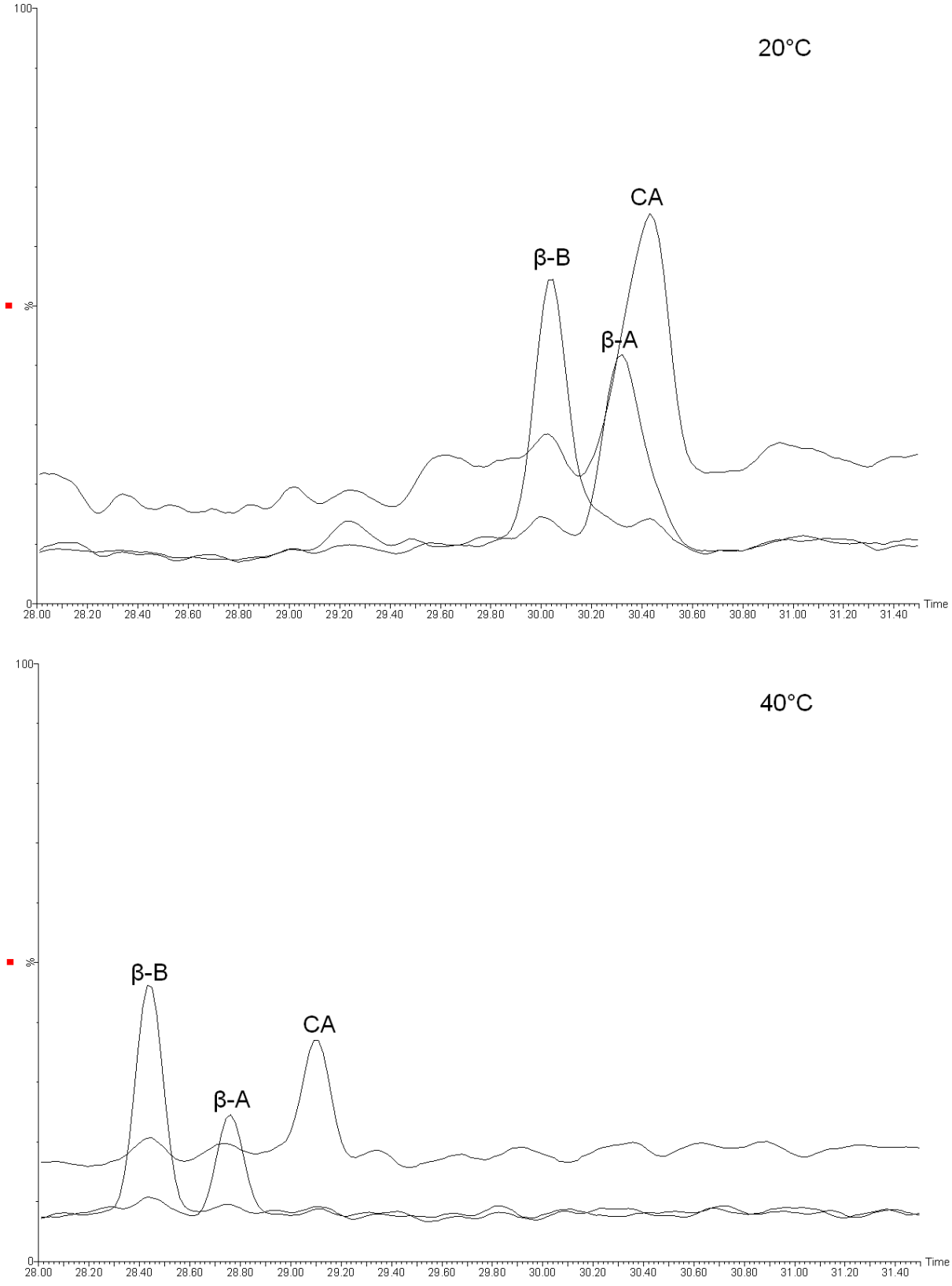

Figure 22. Chromatograms (from 28 min) of mixture of protein standards on PLOT column at column temperature 20°C (top) and 40°C (bottom). Injected concentration was 10 µg/mL of each protein. Other conditions were as in Figure 16. The intensity scale is the same for both temperatures.

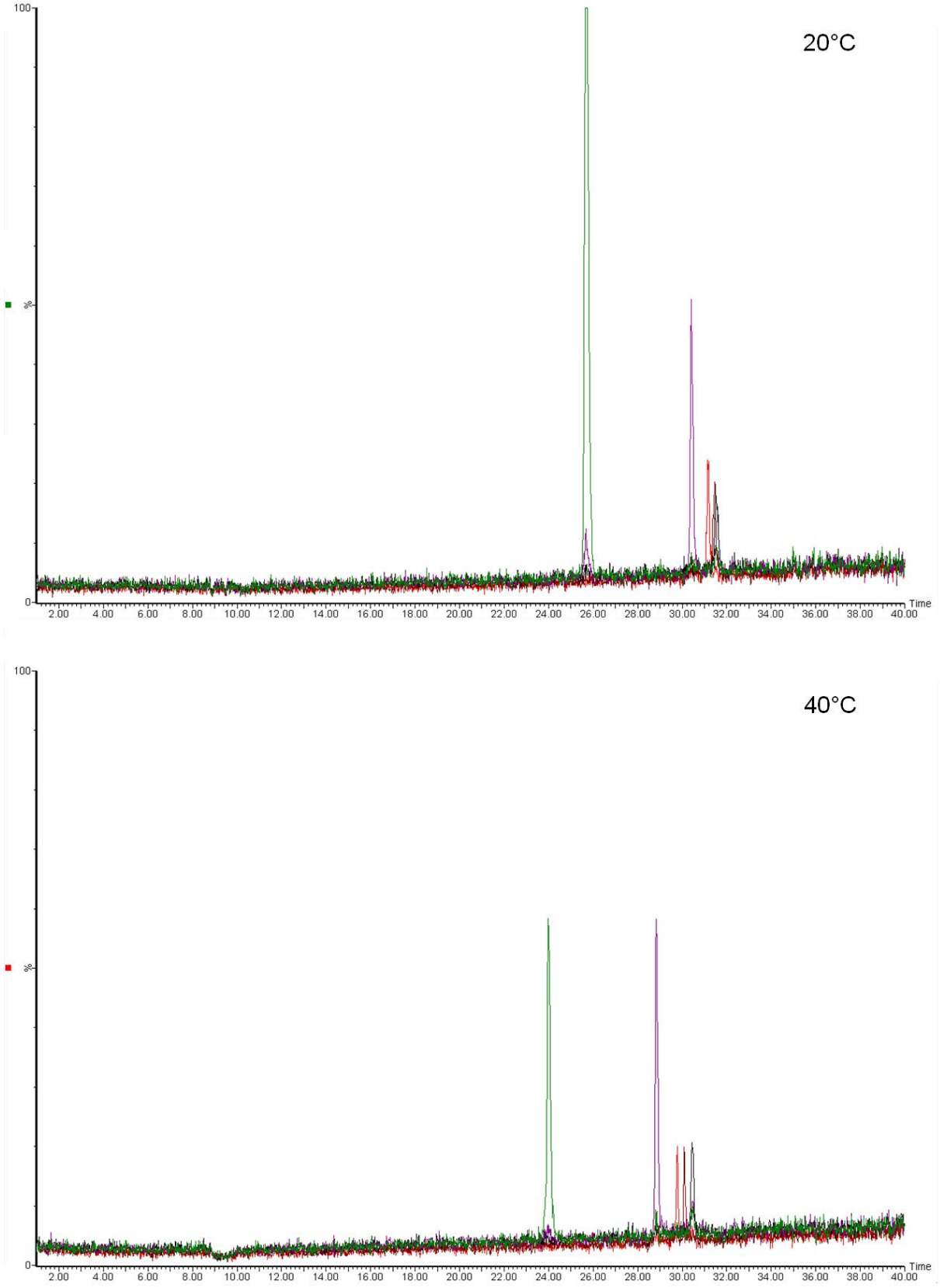

Figure 23. Chromatograms of mixture of protein standards on PLOT column at column temperature 20°C (top) and 40°C (bottom). Injected concentration was 10 µg/mL of each protein. Other conditions were as in Figure 16. The intensity scale is the same for both temperatures.

The relationship of temperature and gradient retention factor (k_g) is illustrated in a Van't Hoff plot in Figure 24. The figure shows a decrease in retention but also an increase in selectivity as a function of temperature. The change in slope of ln k_g at 50° C implies a change in protein or stationary phase conformation at this temperature [76]. A Van't Hoff plot of peptides showed the same trend (Appendix II), suggesting that it is the stationary phase that undergoes change at higher temperatures rather than the proteins.

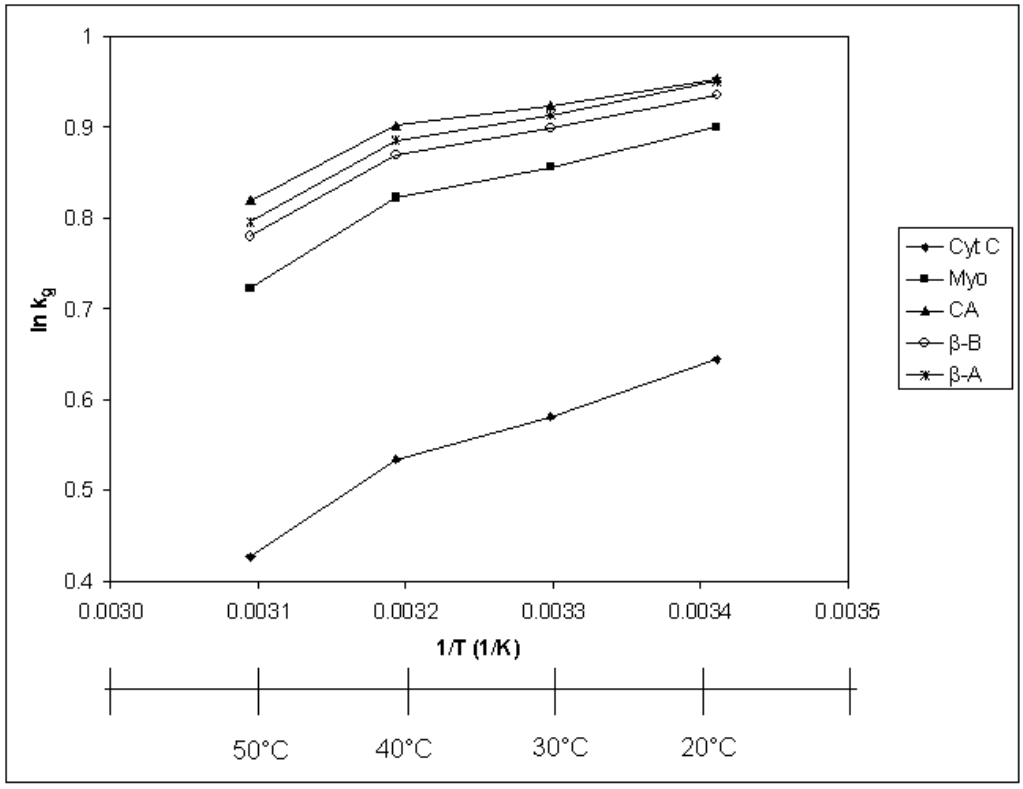

Figure 24. Van't Hoff plot of intact protein separations on PLOT column. Injected concentration was 10 µg/mL of each protein. Conditions were as in Figure 16.

At higher temperatures the peak height decreased as a function of temperature, and at 60°C the signals for the three late eluting proteins were below the limit of detection (LOD) (Appendix II). This effect was also observed without TFA as mobile phase additive (Appendix II), and for smaller peptides (Figure 25), weakening the initial hypothesis that the proteins were adsorbed on the column at increased temperature. Instead, we speculate that the lowering in signal is due to poorer charged droplet formation in the nanospray at elevated temperatures. A similar effect of temperature was reported by Hazotte et al., who found that

the LOD of a ceramide was significantly reduced by increasing the temperature of the incoming solvent to ESI-MS. The opposite effect has been observed with atmospheric pressure chemical ionization (APCI)-MS [77].

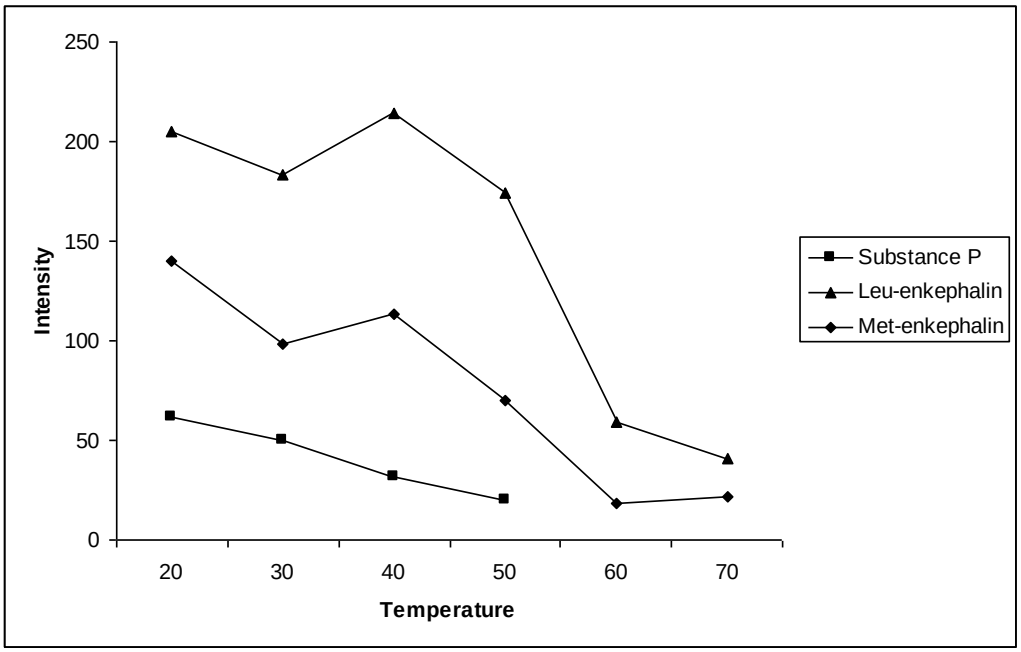

Figure 25. Signal intensity of the peptide peaks vs. temperature. Substance P had no visible signal at 60 and 70°C. The solvent gradient was 100 % A (0.1% FA, 0.05% TFA (v/v) in water) to 40 % B (0.1% FA, 0.05% TFA, 10% water (v/v) in acetonitrile) in 40 minutes. Injected concentration was 1.2, 0.4 and unknown µg/mL of Leu-, Met-enkephalin and substance P respectively.

6.7 Separation of "real" samples

6.7.1 Skimmed milk

A PLOT column was used to separate proteins in skimmed milk (0.1 % fat) (Figure 26). Peak 1 and 2 are unidentified proteins, while 3, 4 and 5 are α-lactalbumin, β-lactoglobulin B and A, respectively. The system performed well in that the lower concentration proteins eluted as narrow and symmetrical peaks, even when the high abundance proteins showed overloading. Identification of α-lactalbumin, β-lactoglobulin B and A, respectively was done by comparison with protein standards (t_R and mass spectrums).

6.7.2 Saliva and blood plasma

A saliva sample and a human blood plasma sample were also injected (results not shown). The saliva sample had to low protein concentration and pre-concentration [78] is necessary. Pepaj et al. used 100 µL of five times diluted saliva sample for protein analysis [79]. As expected the plasma sample was too complex and sample pre-treatment and preferably a 2D-LC system should be used in order to obtain information about the proteins [12,80].

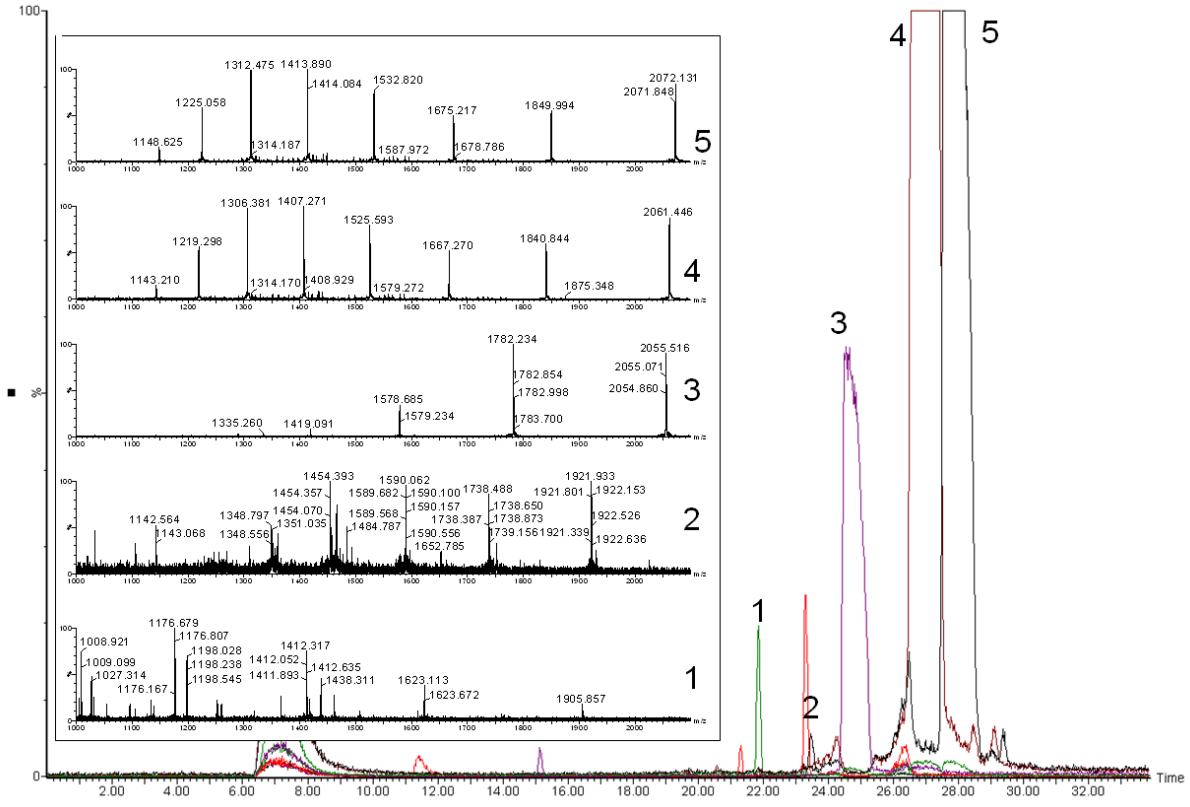

Figure 26. Separation of proteins in skimmed milk. The figure shows an overlay of extracted ion chromatograms of 5 proteins. Conditions were as in Figure 16.

6.8 Assessing the potential of coupling PLOT columns to on-line trypsination

Although we have showed that PLOT columns are well suited for separation of intact proteins, there are limits to this approach and as mentioned earlier, it can be difficult to monitor large proteins with currently available ESI-MS instrumentation. A future goal is

therefore to couple PLOT columns on-line with an immobilized enzyme reactor [81,82], with the intent of separating the intact proteins, immediately digesting them after elution and monitoring the resulting peptides by MS/MS for identification of the proteins. Such an approach will likely require the proteins to be reduced and alkylated at forehand, using a solvent pH of ~7.5-8.5 and a temperature of approximately 40° C for digestion.

The five protein standards did not give any ESI-MS signal at pH 7.8. This is most likely because of too low ionization, so the proteins do not get a sufficient low *m/z* ratio to be observed by the available MS. Thus, in order to study the chromatographic behaviour of reduced and alkylated proteins, a mobile phase pH of 2.4 was used.

6.8.1 Alkylated β-lactoglobulin A and B

As seen in Figure 27 reduced and alkylated β-lactoglobulin A chromatographed with the same high efficiency as the native protein, at pH 2.4. Their mass spectra show mass shift corresponding to complete alkylation of the five cysteine groups found in β-lactoglobulin A. As expected alkylated β-lactoglobulin A and B did not get fully separated at this temperature (Figure 28).

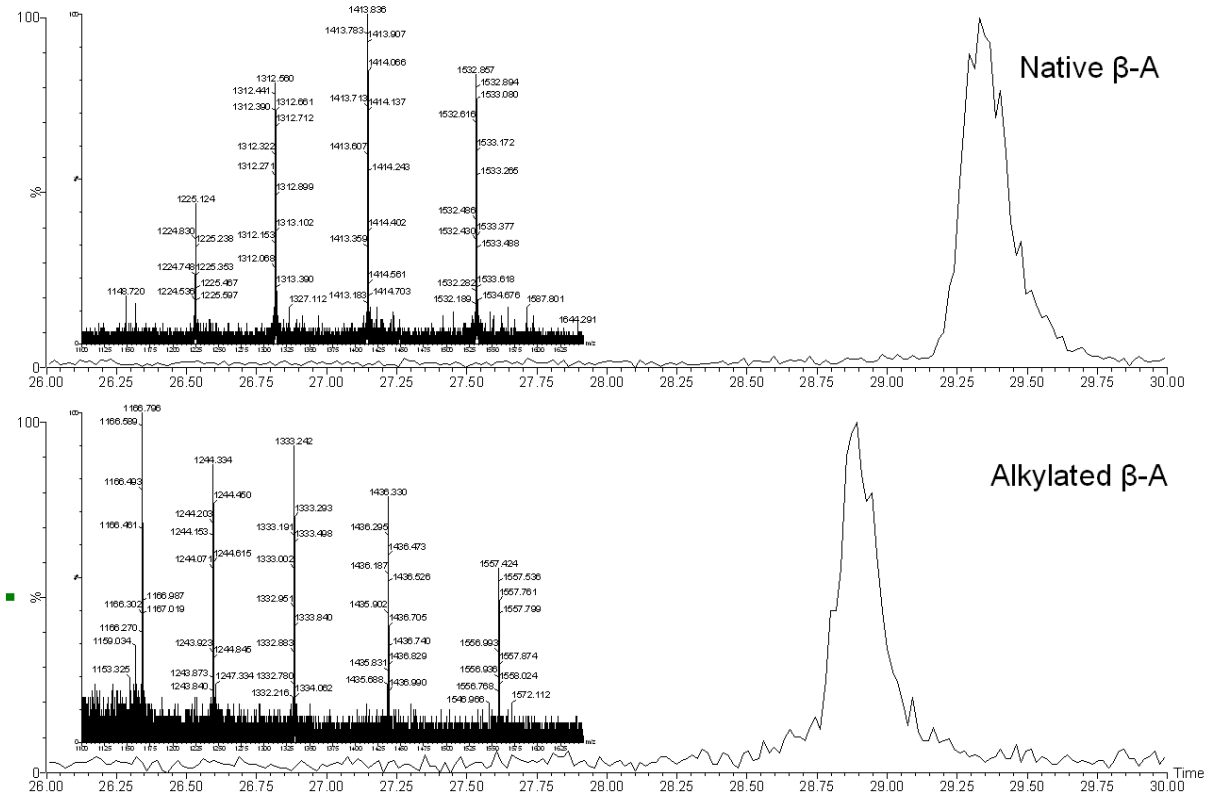

Figure 27. Extracted ion chromatograms of a mixture of alkylated and native β-lactoglobulin A, and their respective mass spectra. Injected concentration was 0.1 mg/mL of each protein. Conditions were as in Figure 16. Each chromatogram and mass spectrum is scaled relative to the highest intensity signal.

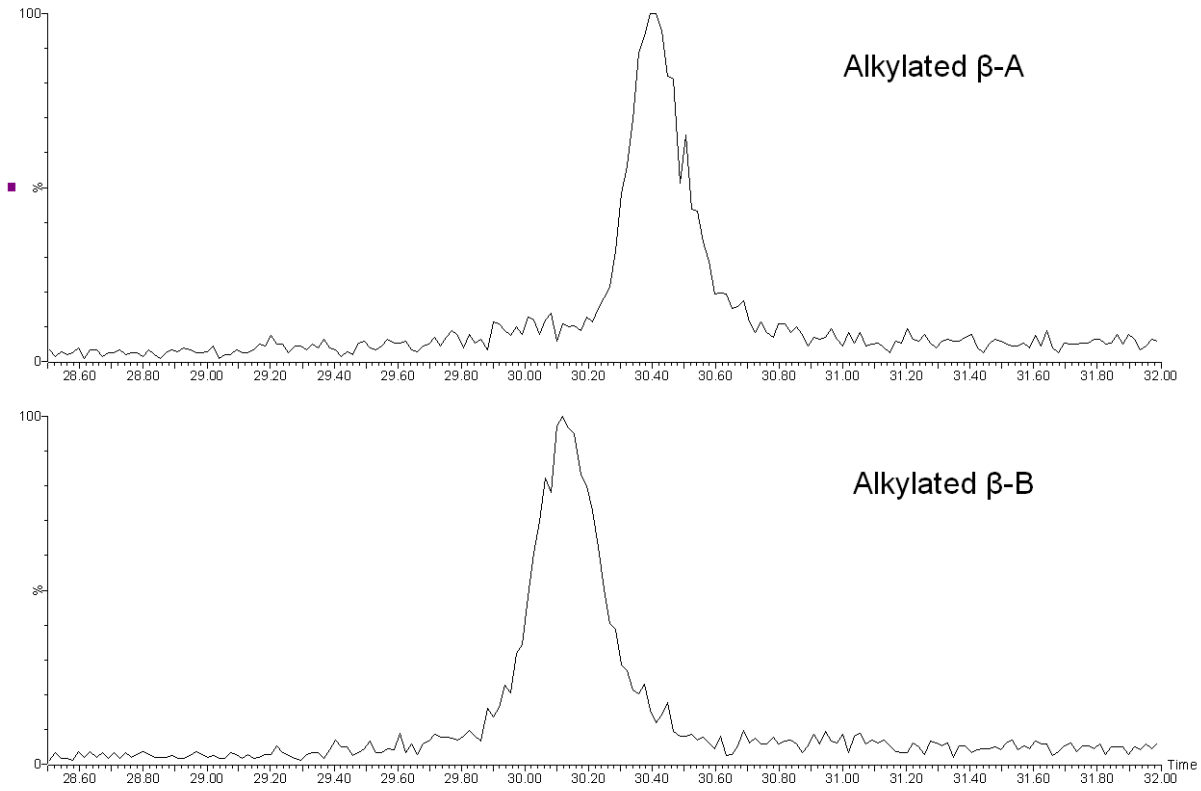

Figure 28. Extracted ion chromatograms of a mixture of alkylated β-lactoglobulin A and B. Injected concentration was 0.1 mg/mL of each protein. Conditions were as in Figure 16. Each chromatogram is scaled relative to the highest intensity signal.

6.8.2 Separation of peptides at pH 7.8

To assess the potential of coupling the PLOT column directly to a PLOT trypsin reactor (not yet available), tryptic peptides from cytochrome C were separated on the PLOT column at pH 7.8 coupled to nanospray-MS. The peptides were used since proteins were difficult to monitor by MS at this pH. Little or no reduction in chromatographic performance compared to separation at pH 2.4 was observed (Figure 29, Figure 30 and Table 6). Compounds corresponding to peak 1 and 2 are peptides with short sequence and did not get sufficient retention on the column at pH 2.4 and eluted almost at zero retention time (t_0). At pH 7.8 peptide number 2 was more retained and eluted as a narrower peak and the retention order changed for peptide 3 and 4, 7 and 8.

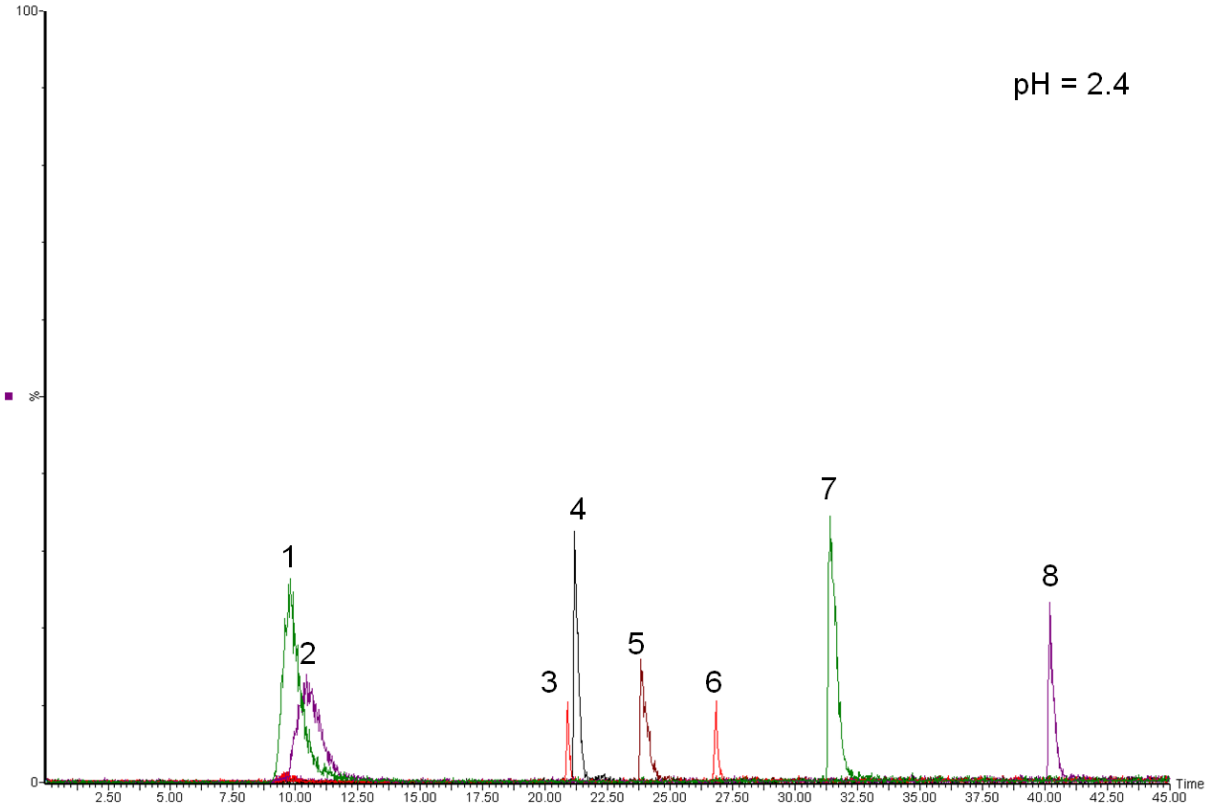

Figure 29. Extracted ion chromatogram of eight peptides from 0.2 mg/mL trypsinated cytochrome C. Injected solution contained 0.2 mg/mL trypsinated cytochrome C. Conditions were as in Figure 25.

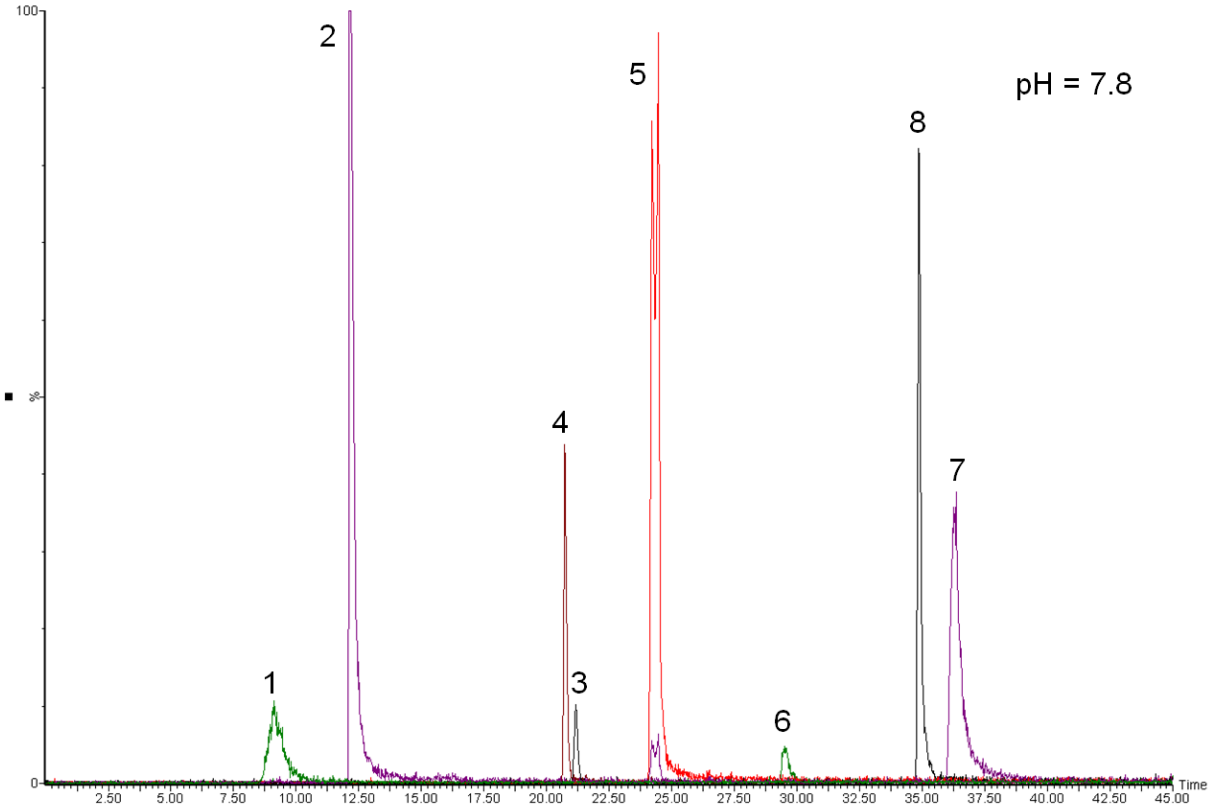

Figure 30. Extracted ion chromatogram of eight peptides from 0.2 mg/mL trypsinated cytochrome C. The solvent gradient was from 100 % A (20 mM ammonium acetate in water, pH 7.8) to 40% B (20 mM ammonium acetate, 10% water (v/v) in acetonitrile) in 40 minutes. The intensity scale is the same as in Figure 29).

Table 6. Peak widths for 4 selected peptides, from Figure 29 and Figure 30.

	pH = 2.4		pH = 7.8	
Peptide	t_R	$w_{0.5}$	t_R	$w_{0.5}$
3	20.89	0.11	21.17	0.15
4	21.17	0.20	20.73	0.13
5	23.83	0.27	24.33	0.37
6	26.84	0.11	29.50	0.27

7. CONCLUSION

The 10 µm i.d. PS-DVB PLOT columns performed well in separation of intact proteins, with regard to peak shape, carry-over and repeatability; hence these columns have a potential for separation of more complex samples, as encountered in e.g. top-down proteomics. The system performed well for separating proteins in a "real" sample like milk. The columns were prepared following the methodology of Karger's group [27], which was easily reproduced and understandable. In addition, PLOT columns performed well under conditions that an on-line tryptic digestion system will require.

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9. APPENDIX I

9.1 Protein separation on packed columns

Carry-over for large peptides and proteins are largely dependent upon the stationary phase used [20]. Before examination of the carry-over for the new reversed phase (RP) surface porous Halo® C18 particles, a packing procedure for micro columns was established. When repeatable columns could be made, a comparison with the much used packing material Kromasil® C18 was carried out in order to determine any benefits regarding carry-over for the new Halo C18 particles.

9.2 Introduction

9.2.1 Superficial porous particles

The concept of shell particles made of a solid core wrapped in a porous shell was pioneered by Horvath [83,84] and Kirkland [85]. Every time in the last 40 years a new type of shell particles became available, their advantages became rapidly outcompeted by smaller fully porous particles, which eliminated the advantages of the shells. Recently, new shell particles were introduced [86]. These Halo 2.7 µm particles have a 0.5 µm thick porous shell. The particle size was chosen to achieve both high efficiency and sufficiently low permeability to be used on conventional HPLC instruments (<400 bar). There are three main reasons for the higher efficiencies obtained with these Halo particles [87]. (1) The axial diffusion can be reduced by decreasing the intra-particle pore volume. The solid core and their proprietary preparation methods give an intra-particle pore volume of 0.15 instead of 0.35-0.40 for conventional packing materials. (2) Eddy dispersion can be reduced by decreasing particle size distribution (PSD). The PSD of Halo particles are only 5% instead of 10-20% for regular porous silica. (3) Mass transfer through particles is enhanced by reducing diffusion length, e.g. smaller particles and possibly shell particles.

Gritti and Guiochon performed a comparative study on the performance of several columns packed with new silica particles including Halo, over a wide range of mobile phase velocities. The Halo column performed best for small compounds (e.g. naphthalene), but not

as good for the large peptide insulin. The high C-term at increased mobile phase velocities was explained by the high surface roughness, which might generate a high film mass transfer resistance [87].

Even though several papers have described separation of peptides and proteins on the new columns packed with Halo particles [87-90], carry-over issues have not been addressed.

9.2.2 Packing columns

Most of the packing procedures of columns in liquid chromatography are using slurry techniques [91]. This slurry packing procedure usually involves: a packing solvent reservoir, a high pressure pump, a slurry reservoir and the HPLC column body (Figure 31) [33]. The slurry is made by sonication of a mixture of the particles and slurry-liquid. The reservoir is filled with the slurry-liquid and connected to the pump. The slurry is forced into the empty column by the packing liquid. At the outlet side of the column a porous frit is placed that allows liquid to flow through while retaining the packing material. When the packing is finished, the column is detached and another porous frit is placed on the open inlet of the column [33]. Several packing- and slurry-liquids have been studied in order to make reproducible and stable high performance columns [91].

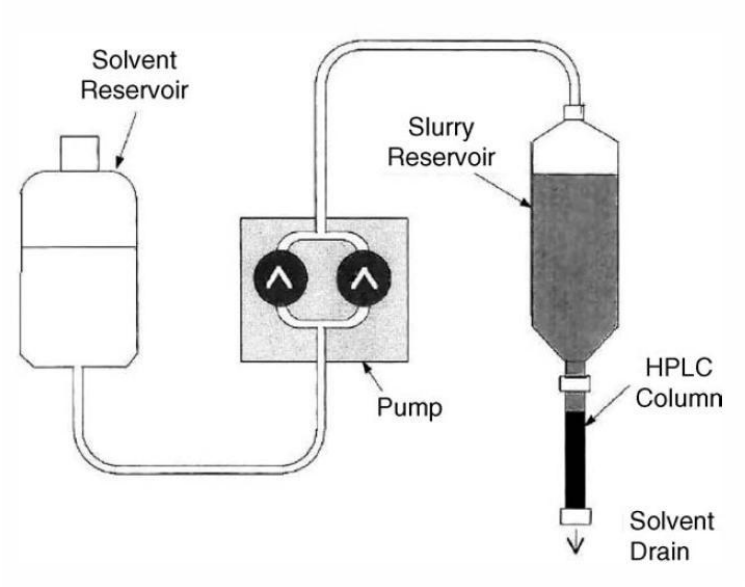

Figure 31. A standard setup for column filling using the slurry technique. Figure reprinted from [33].

9.3 Experimental

Also see main thesis for location of suppliers.

9.3.1 Procedure for slurry packing columns

Columns of 15 cm × 0.3 mm i.d. were packed in-house with a downward high pressure liquid slurry method, using a mixture of acetonitrile/water (70/30, v/v) as packing liquid. The slurry liquid was carbon tetrachloride (99%) (VWR, Oslo, Norway) if not otherwise stated, and the packing material was either Kromasil C18 (G&T Septech, Kolbotn, Norway) or Halo C18 (Advanced Materials Technology, Wilmington, DE). A union (Valco) with a steel frit (Valco) was connected to one end of the steel column, and the other end was connected to the packing chamber. The slurry liquid was ultrasonificated for 10 minutes and then transferred to the packing chamber with a 1 mL plastic syringe coupled to a fused silica tubing (~10 cm × 320 µm i.d.). An ISCO 100 DM syringe pump (Lincoln, NE) with an ISCO series D pump controller was subsequently connected to the packing chamber. The pressure was increased to 100 bar by opening a valve, then increased up to 650 bar at a rate of 100 bar/min. The column was subjected to 650 bar for 15 minutes, before it was depressurized for 30 minutes. The column was then detached from the packing chamber, and a filter (1 and

2 µm used to pack Halo C18 and Kromasil C18 particles respectively) (Valco) and a union were connected to the free end of the steel column.

9.3.2 Column efficiencies

The columns were evaluated using a LC-UV system equipped with an Agilent 1100 series pump with a G1379A series degasser, and a Linear UV-Vis 200 UV detector (Spectra-Physics, San Jose, CA). UV detection was performed at 254 nm. A ~15 cm × 50 µm i.d. capillary was used to connect the injector with the column, and a 75 µm i.d. capillary with a detection window served as a on-column measuring cell (~25 cm from end of column to measuring point). Mobile phase composition was 80% B, where mobile phase A was 90/10 water/acetonitrile (v/v) and mobile phase B was 90/10 acetonitrile/water (v/v). Injection was done by using a 500 nL injector (Valco), and a 0.1 mg/mL solution of toluene in water was used as standard. Data acquisition was acquired by TotalChrom version 6.2.1 (PerkinElmer, Waltham, MA) at 1 sampling per second. Each value shown is the average from three replicates. Column efficiencies were calculated by a built-in method in TotalChrom using the tangential method.

Other packing liquids used were methanol (Merck), acetone (Merck), acetonitrile (Rathburn) and acetonitrile/water 70/30 (v/v).

9.3.3 Column carry-over

For carry-over investigation 5 µL protein solution (0.1 mg/mL) was injected into the column using an injector (Valco) with a 5 µL external polyetherether ketone (PEEK) (Upchurch Scientific Inc, Oak Harbour, WA) loop. Proteins used are shown in Table 7. Detection was performed at 220 nm. A gradient from 5-90% B in 17 minutes was used, with mobile phase A being 0.1% TFA (v/v) in water and mobile phase B being 0.1% TFA (v/v) in either acetonitrile or methanol. For temperature control a Mistral LC-oven was used. Each injection of protein standard was followed by one or more injections of a blank (water). Injected concentration was 0.1 mg/mL of each protein. Each value shown is the average for two replicates.

Table 7. Proteins and masses for the proteins used for carry-over investigation on columns packed with Kromasil or Halo particles.

Protein	Mass (kDa)
Cyt C	12
β -B	18
Ova	44
BSA	66

9.4 Results and discussion

9.4.1 Evaluation of slurry liquids

In order to test protein carry-over in columns packed with Halo particles, an appropriate packing procedure had to be established. First the appropriate slurry liquid had to be found, since the in-laboratory packing procedure had not been used for Halo particles previously. Different slurry liquid compositions were examined, while the packing liquid was kept the same. The different slurry liquids were chosen from a slurry-packing study of Vissers et al. [91]. The same slurry liquid as used for Kromasil C18 particles gave the best chromatographic efficiency for the columns packed with Halo particles (Figure 32).

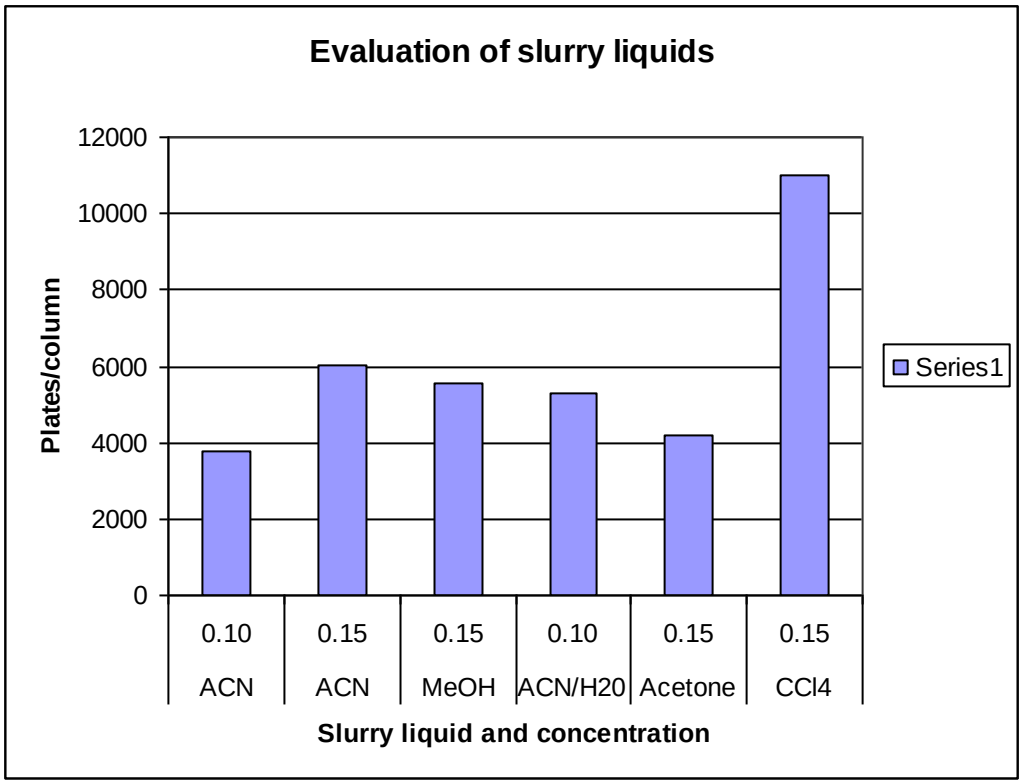

Figure 32. Column efficiency of 15 cm × 0.3 mm i.d. columns packed with Halo C18 particles as a function of slurry liquid and slurry concentration (w/v). Toluene was used for measuring the plate number.

9.4.2 Packing repeatability

In order to determine if the packing procedure using carbon tetrachloride as slurry liquid and acetonitrile/water 70/30 (v/v) as packing liquid was satisfactory; a comparison with a Kromasil C18 column was made. Table 8 shows retention times, plates/meter and the tailing factor for 3 columns packed with both materials. These results were considered sufficient for protein carry-over investigation.

Table 8. Repeatability for three columns packed with Halo C18 or Kromasil C18 particles using CCl4 as slurry liquid.

Kromasil	t_R (min)	N (plates/meter)	Tailing
Col 1	4.76	1.5E+04	1.24
Col 2	4.60	1.5E+04	1.31
Col 3	4.70	1.4E+04	1.43
Halo			
Col 1	3.35	1.1E+04	1.23
Col 2	3.69	1.1E+04	1.27
Col 3	3.62	1.1E+04	1.34

9.4.3 Carry-over

Three protein standards were analyzed separately on each column (Figure 33 and Figure 34), followed by injection of water. Both columns performed well under the chromatographic conditions, and little or no difference was seen in column performance, i.e. peak widths and asymmetry.

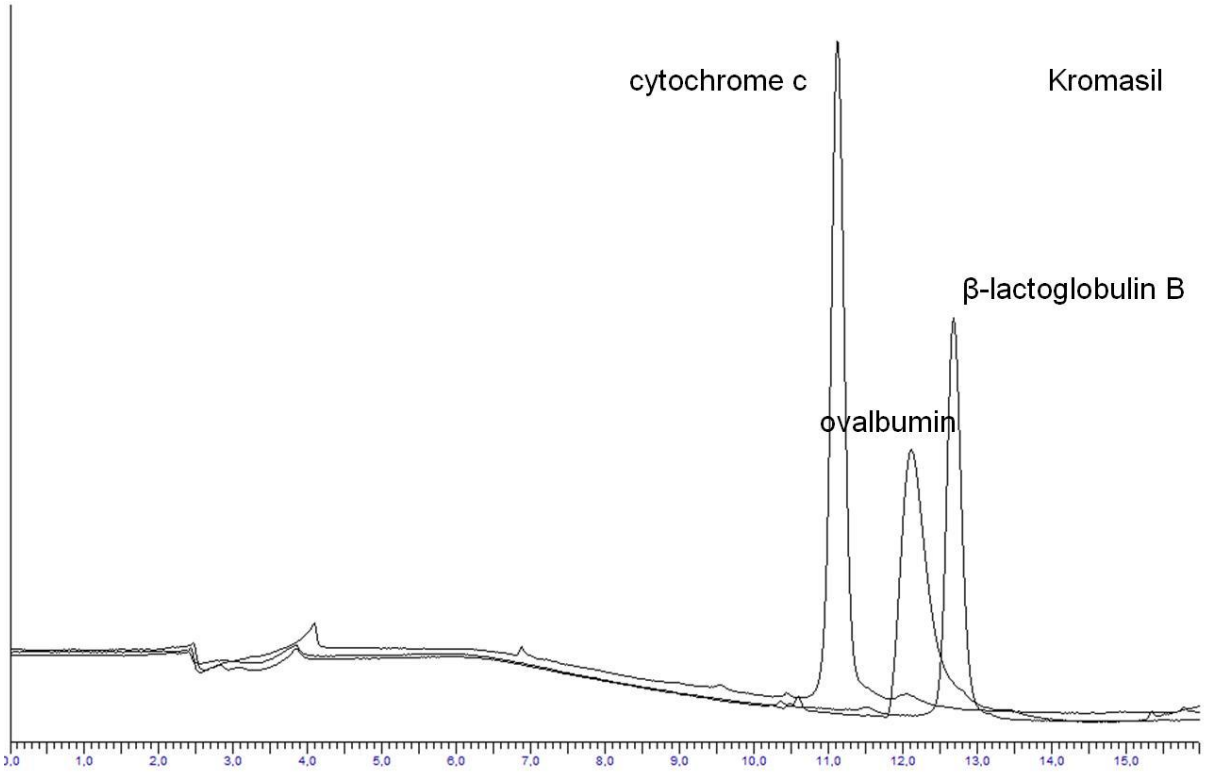

Figure 33. Overlay of chromatograms of cytochrome C, ovalbumin, and β-lactoglobulin at 40°C on a 15 cm × 0.3 mm i.d. packed in-house with Kromasil C18 particles. The solvent gradient was from 95% A (0.1% TFA (v/v) in water) to 90% B (0.1% TFA (v/v) in acetonitrile) in 17 minutes. UV-detection at 220 nm.

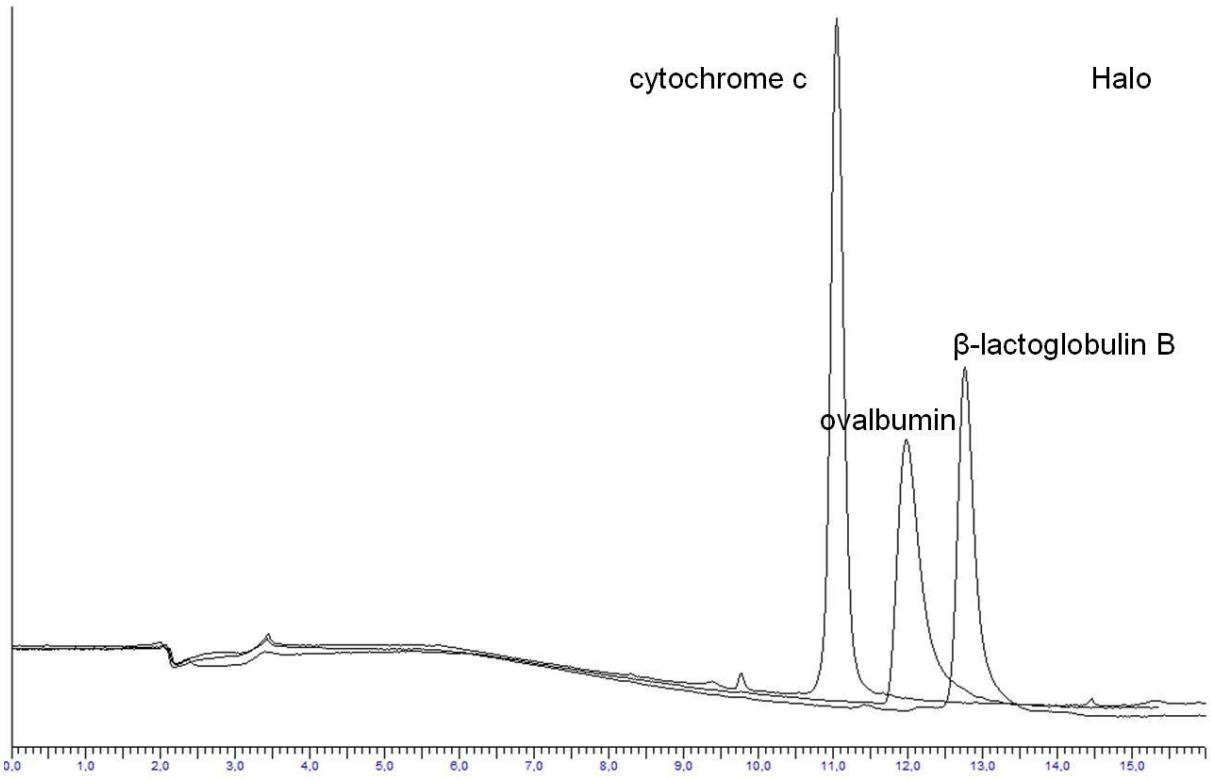

Figure 34. Overlay of chromatograms of cytochrome C, ovalbumin, and β-lactoglobulin 40°C on a 15 cm × 0.3 mm i.d. packed in-house with Halo C18 particles. Conditions were as in Figure 33.

Little difference in protein carry-over between the two columns was found (Figure 35 and Figure 36). The temperature effect was also small. However, to fully see the effect of temperature more temperatures over a wider range should be examined.

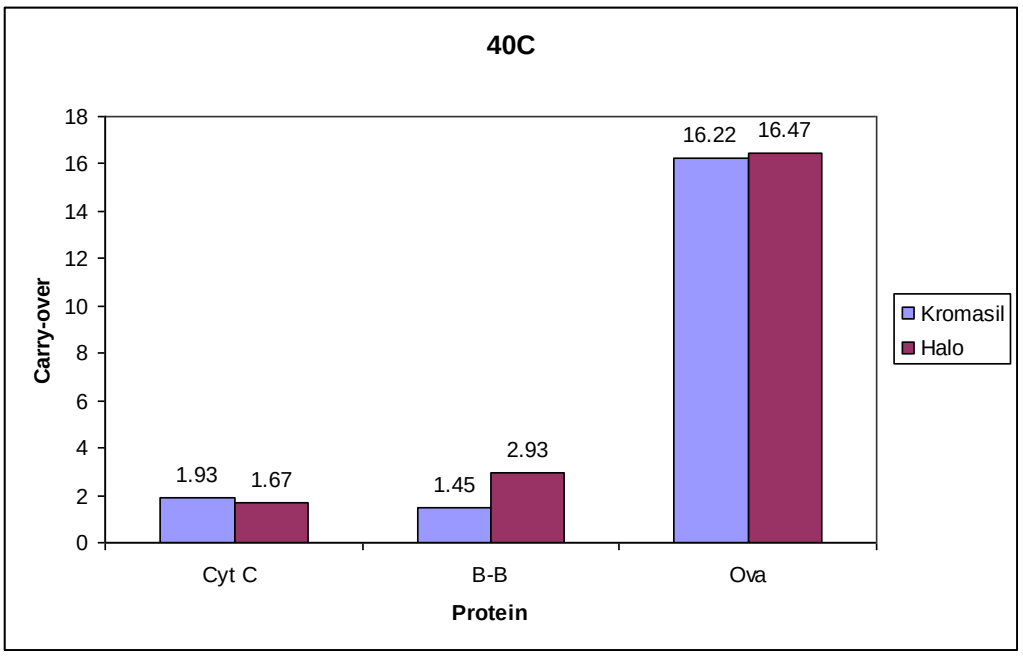

Figure 35. Carry over for cytochrome C, β-lactoglobulin B and ovalbumin at 40°C. Conditions were as in Figure 33.

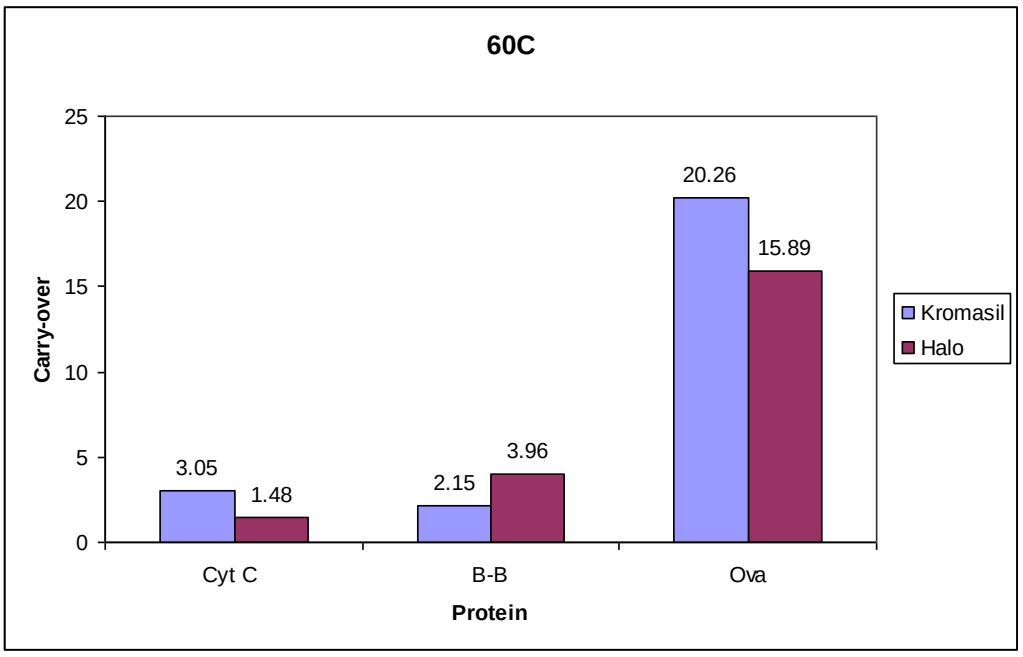

Figure 36. Carry over for cytochrome C, β-lactoglobulin B and ovalbumin at 60°C. Other conditions were as in Figure 33.

9.4.4 Additional carry-over data for the Kromasil C18 column

Different proteins over a wide range of molecular masses were separated with methanol as mobile phase (due to the global acetonitrile shortage) at 40 and 60°C. As seen in Figure 37 larger proteins show more carry-over.

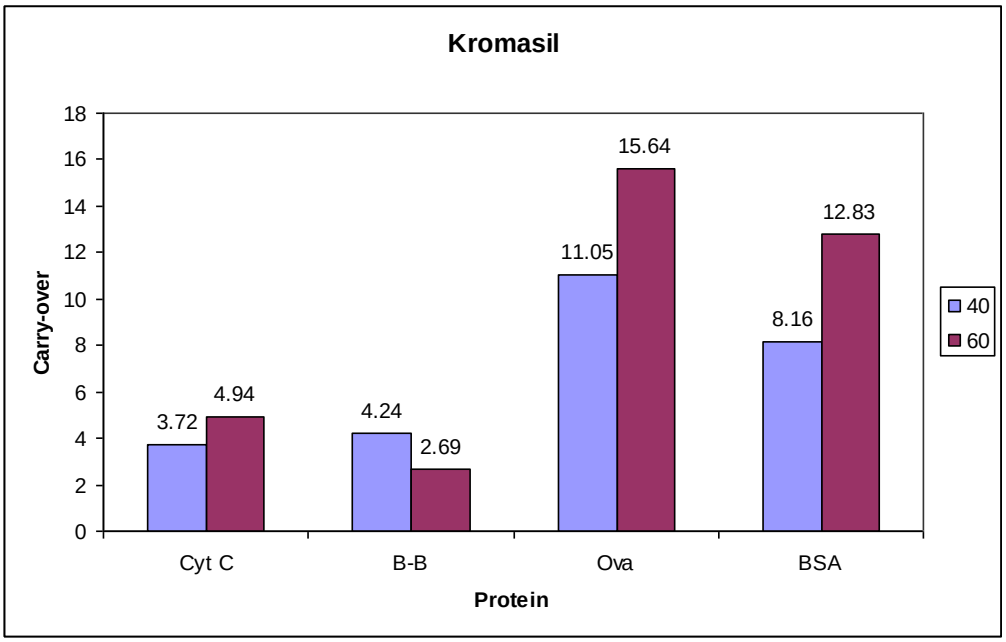

Figure 37. Carry-over on a column packed with Kromasil C18 particles at 40 and 60°C with methanol as mobile phase. Protein size increasing from left to right. Other conditions were as in Figure 33.

More than one blank injection was performed for the larger proteins in order to remove carry-over from previous injections and also to get information about the carry-over for several blanks (Figure 38). The third subsequent blank of BSA was too small for accurate determination at 40°C, hence only two blanks were injected at 60°C. Carry-over also increased for BSA and Ova at higher temperatures when methanol was used in the mobile phase.

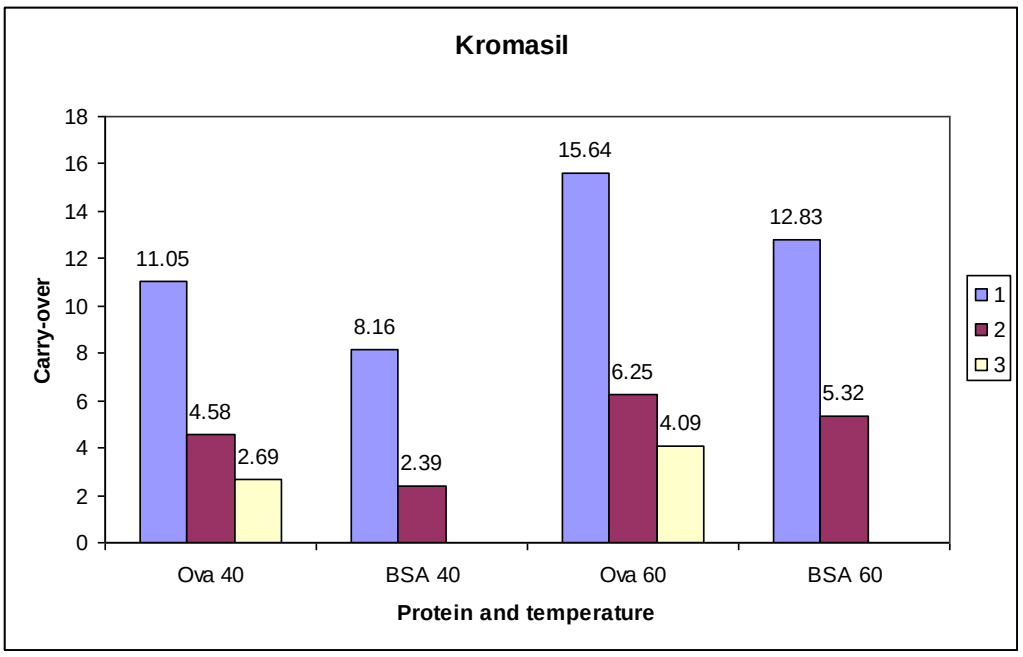

Figure 38. Carry-over as a function of number injected blanks at 40 and 60°C after the protein injection. Conditions were as in Figure 33.

10. Appendix II

10.1 Additional figures

10.1.1 gradient time and column length

The effect of gradient time and column length is shown in Figure 39-41.

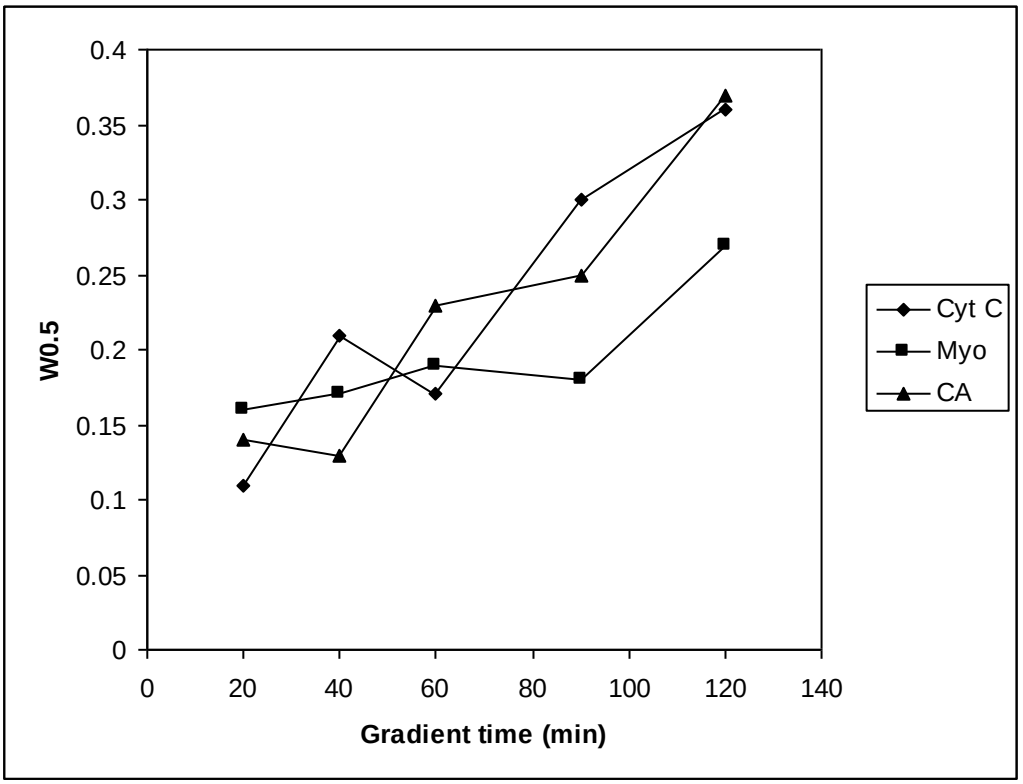

Figure 39. Plot of the average relative $w_{0.5}$ as function of t_G on 3 meter PLOT column. Injected concentration was 33 µg/mL of each protein. Other conditions were as in Figure 16.

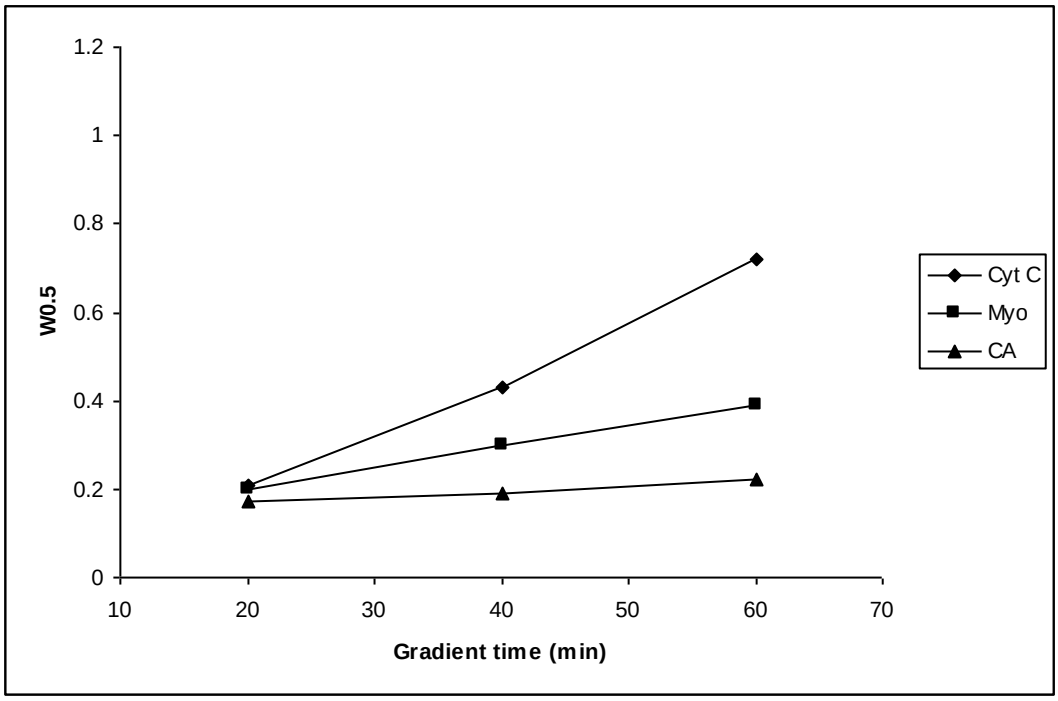

Figure 40. Plot of the average relative $w_{0.5}$ as function of t_G on 2 meter PLOT column. Injected concentration was 33 µg/mL of each protein. Other conditions were as in Figure 16.

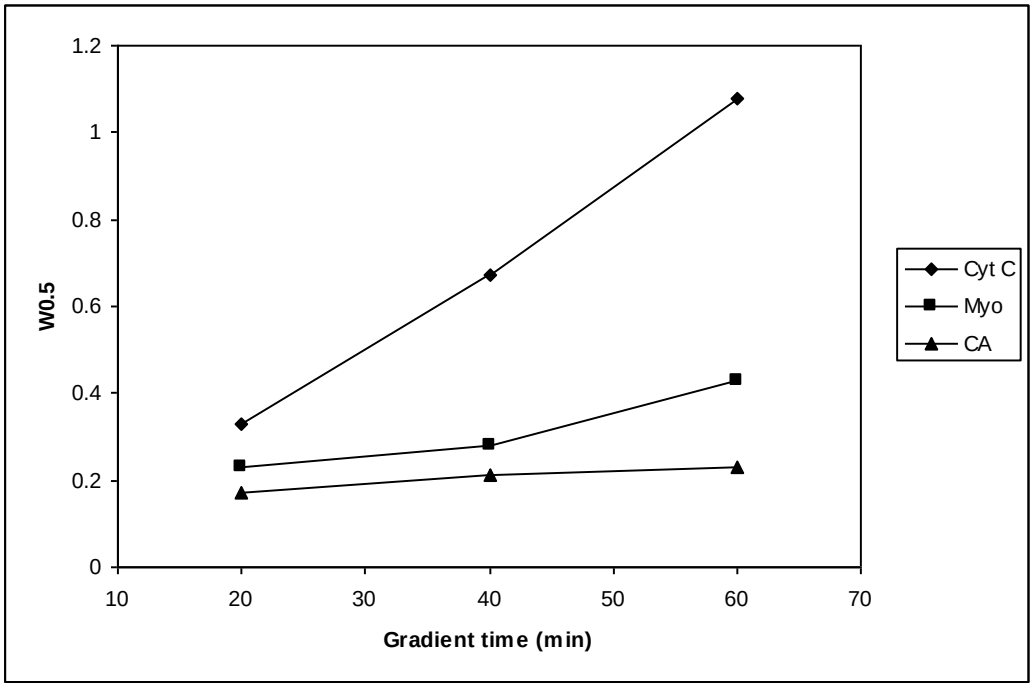

Figure 41. Plot of the average relative $w_{0.5}$ as function of t_G on 1 meter PLOT column. Injected concentration was 33 µg/mL of each protein. Other conditions were as in Figure 16.

10.1.2 Van't Hoff plot on peptide separation

The relationship of temperature and gradient retention factor (k_g) is illustrated in a Van't Hoff plot in Figure 42.

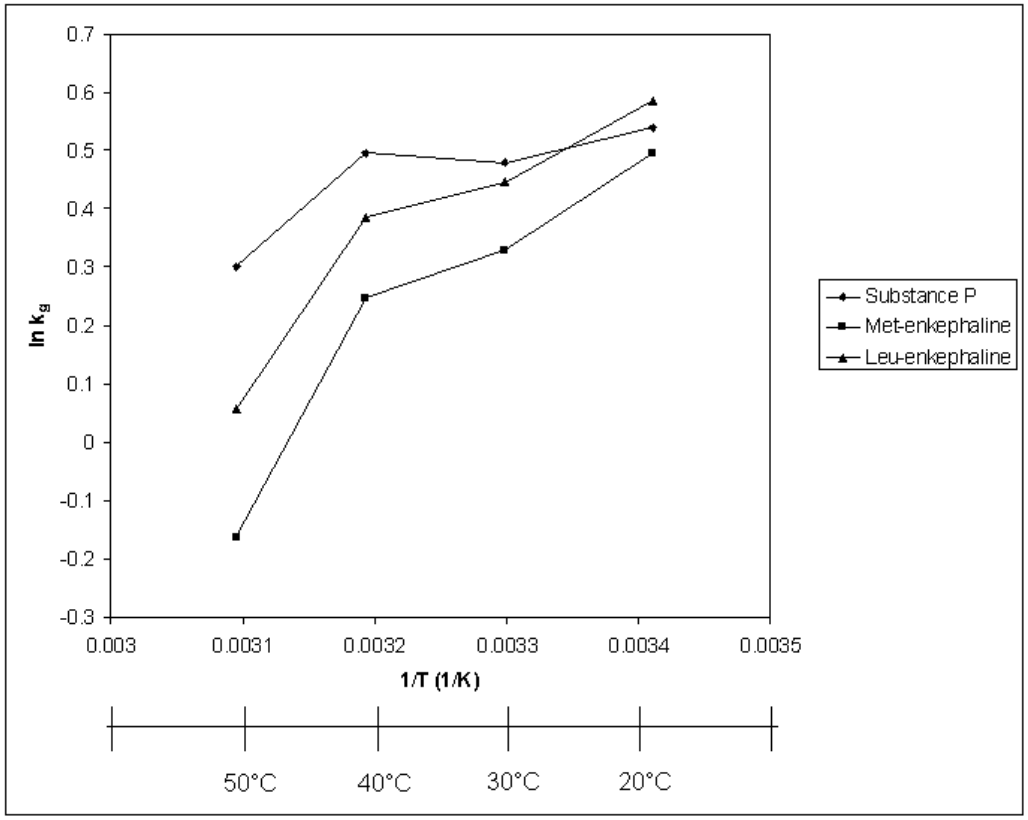

Figure 42. Van't Hoff plot of peptide separations on 3 m PLOT column at 20, 30, 40 and 50°C. Injected concentration was 1.2, 0.4 and unknown µg/mL of Leu-, Met-enkephalin and substance P respectively. Other conditions were as in Figure 25.

10.1.3 Separation with TFA in the mobile phase at 60°C

The effect of temperature on signal intensity at 60°C is seen in Figure 43.

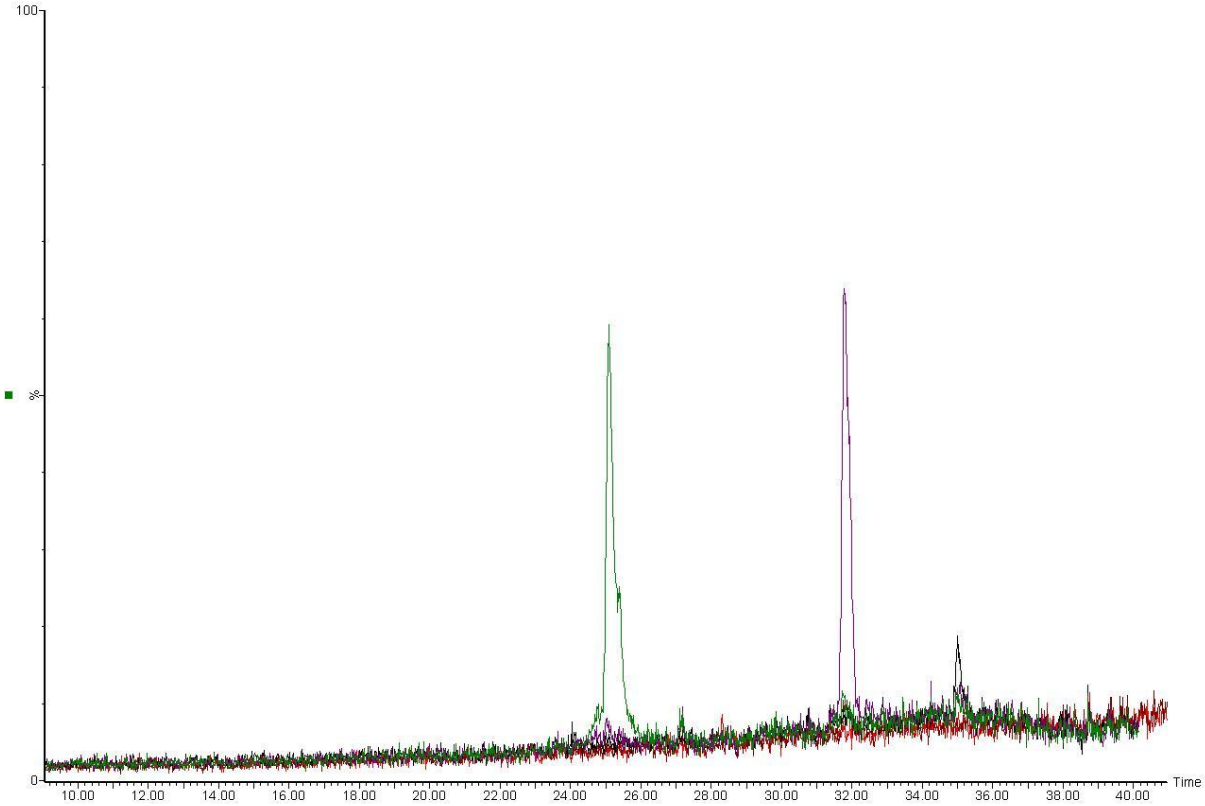

Figure 43. Extracted ion chromatogram of five protein standards separated on a PLOT column at 60°C. Injected concentration was 10 µg/mL of each protein. The solvent gradient was 90 % A (0.1% FA, 0.05% TFA (v/v) in water) to 90 % B (0.1% FA, 0.05% TFA, 10% water (v/v) in acetonitrile) in 40 minutes.

10.1.4 Signal intensity at elevated temperatures

As seen in Figure 44-47 the signal intensity decreased at elevated temperatures.

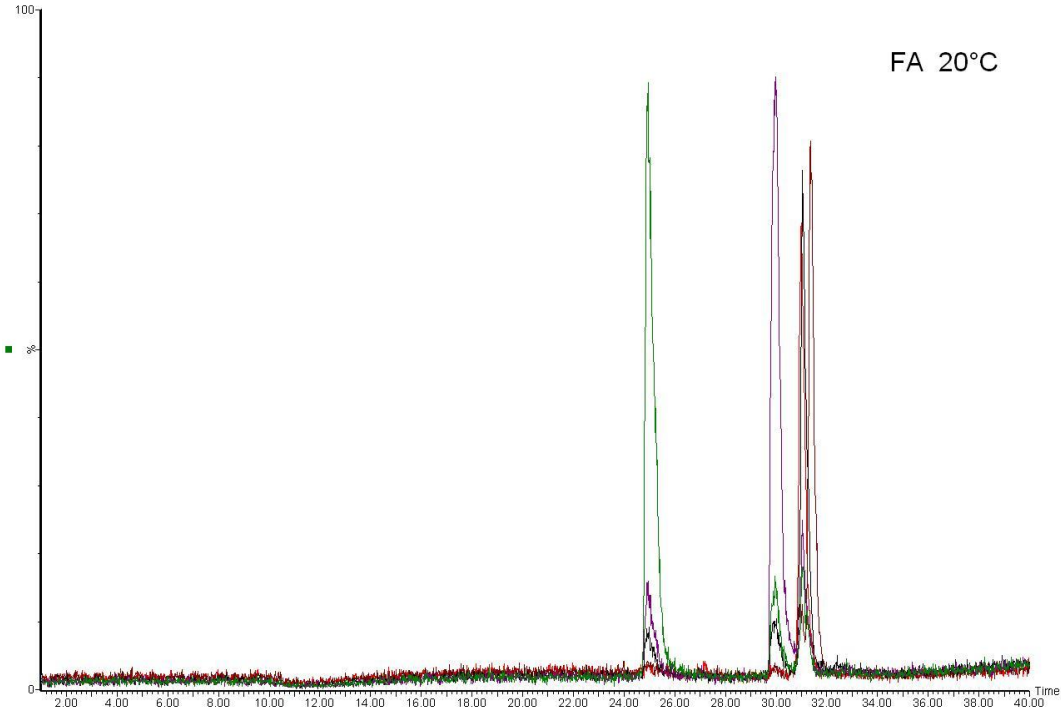

Figure 44. Extracted ion chromatogram of five protein standards separated on a PLOT column at 20°C. Injected concentration was 10 µg/mL of each protein. The solvent gradient was 90 % A (0.1% FA (v/v) in water) to 90 % B (0.1% FA, 10% water (v/v) in acetonitrile) in 40 minutes.

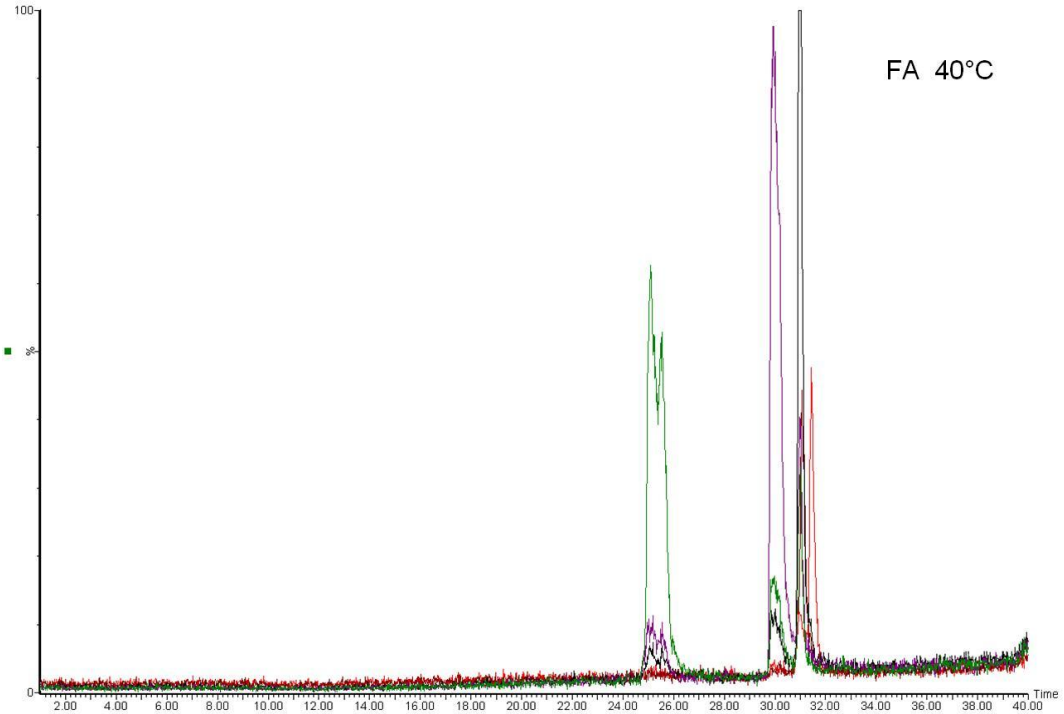

Figure 45. Extracted ion chromatogram of five protein standards separated on a PLOT column at 40°C. Injected concentration was 10 µg/mL of each protein. Other conditions were as in Figure 44.

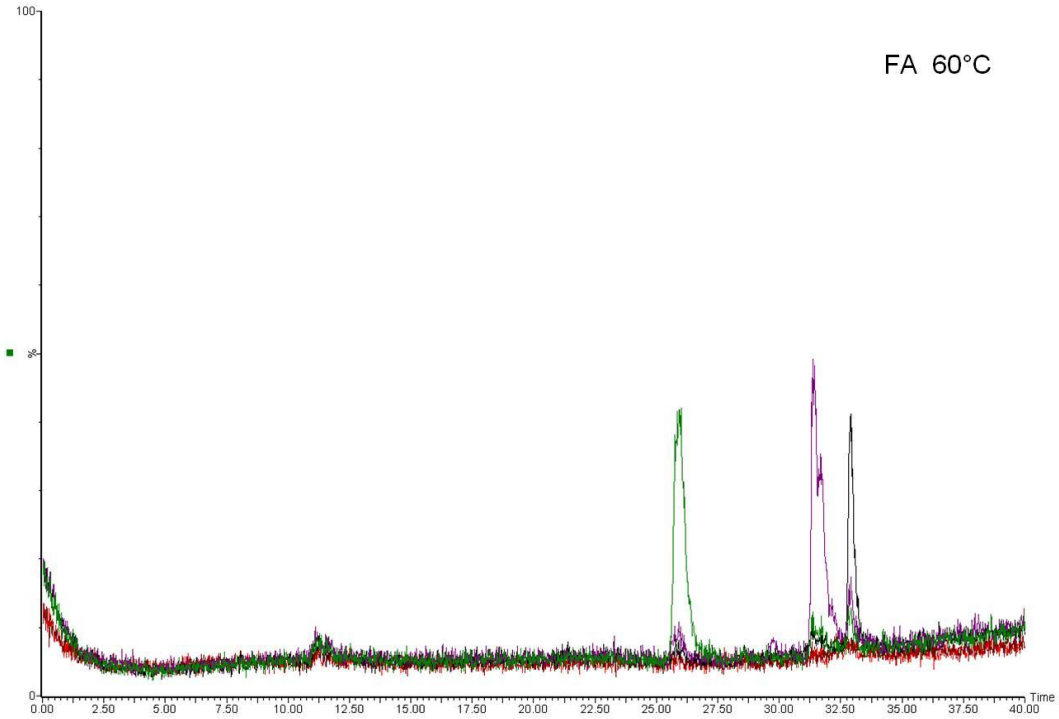

Figure 46. Extracted ion chromatogram of five protein standards separated on a PLOT column at 60°C. Injected concentration was 10 µg/mL of each protein. Other conditions were as in Figure 44.

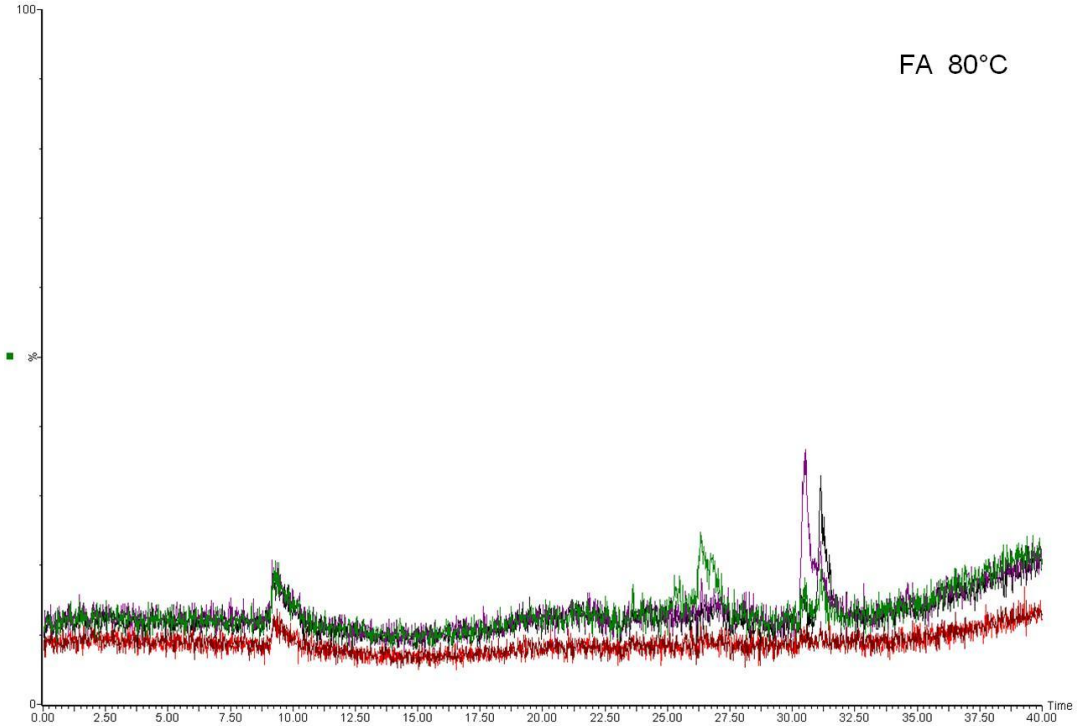

Figure 47. Extracted ion chromatogram of five protein standards separated on a PLOT column at 80°C. Injected concentration was 10 µg/mL of each protein. Other conditions were as in Figure 44.

10.2 Additional information

The *m/z* values used to produce extracted ion chromatograms are shown in Table 9.

Table 9. Extracted ion chromatogram *m/z* values.

Carbonic Anhydrase	741.1+837.1+951.6+1007.8+1087.1+1175.2
Myoglobin	694.2+848.7+893.4+943.1+998.4+1060.6
b-lactoglobulin A	1148.7+1225.1+1312.6+1414.1+1553.2+1675.5
b-lactoglobulin B	1143.0+1219.0+1306.2+1407+1525.4
Cytochrome C	777.5+816.2+874.5+941.7+1020.0+1112.4
β-lactoglobulin A native	1312.6
β -lactoglobulin A alkylated	1333.2
β -lactoglobulin B alkylated	1327.1